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Charakterisierung der frühen adaptiven zerebralen Arteriogenese

Direkter Nachweis der Bradykinin Rezeptor Signalwirkung auf die Arteriogenese

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Zusammenfassung

Arteriogenese bezeichnet das adaptive Wachstum von präexistenten kollateralen Arterien. Im Falle eines Arterienverschlusses ist Arteriogenese der endogen effizienteste Kompensationsmechanismus, um das Hypoperfusionsgebiet mit ausreichend Blut zu versorgen (Biologischer Bypasses). In dieser Arbeit wurde das frühe Wachstum von Kollateralgefäßen im Gehirn im ersten Modell für zerebrale Arteriogenese, dem 3-VO Modell (3-vessel occlusion), in der Ratte charakterisiert.

(I) Die Untersuchung am nicht-ischämischen 3-VO Hypoperfusionsmodell zeigten, dass 7 Tage nach 3-VO die Arteria cerebri posterior (PCA) signifikant im Diameter anwächst. Histologische Untersuchungen konnten ein vermehrtes Zellwachstum in der PCA und das Einwandern von Makrophagen in den perivaskulären Bereich (24 Stunden und 3 Tage post 3-VO) darstellen und eine Aktivierung des Endothels 3 Tage nach 3-VO wurde mittels Rasterelektronenmikroskopie identifizieren.

(II) Für eine genaue Anaylse des globalen Genexpressionsprofils der zerebralen Arteriogense wurde die wachsende PCA selektiv aus dem Gehirn entnommen und ein Genexpressionsprofil für die frühe zerebrale Arteriogenese erstellt (164 Gene dereguliert). Eine Unteruschung von biologischen und molekularen Prozessen zeigte, dass eine Vielzahl der deregulierten Gene in Zellproliferation und Inflammation involviert sind. Die Expression der Protease-Inhibitoren Kininogen und TIMP-1 wurde als “Marker” der frühen Arteriogenese in der PCA lokalisiert werden.

(III) Eine funktionelle Relevanz der Kininogen-Bradykinin-Signalwirkung für die Arteriogenese wurde im Arteria Femoralis Ligatur Modell am Mäusehinterlauf untersucht und zeigte, dass sowohl in Bradykinin Rezeptor (BR1) knockout (KO), in BR2 KO, und in B1R/BR2 doppel-KO Mäusen die Arteriogenese signifikant reduziert ist.

Zusammenfassend zeigt diese Arbeit erstmals eine Übersicht der biologischen Prozesse in der zerebralen Arteriogenese und eröffnet neue Ideen für eine mögliche therapeutische Strategie.

Schlagwörter: Arteriogenese, Gehirn, Genexpression, Kininogen, Bradykinin

Abstract

Arteriogenesis, the adaptive outward growth of pre-existing collateral arteries, is the most efficient endogenous rescue mechanisms in vertebrates against the occlusion of a major artery (biological bypass). Here, collateral growth was induced using the first model for cerebral arteriogenesis, the 3-vessel occlusion (3-VO) rat model.

(I) 3-VO resulted in a significant diameter increase within 7 days in the posterior cerebral artery (PCA) and posterior communicating artery (Pcom), classifying the region of interest. Immunhistological staining demonstrated proliferative activation and macrophage invasion, already 24h post 3-VO within the PCA, confirming the arteriogenic phenotype. Furthermore, activation of the PCA endothelium was detected within 3 days post 3-VO by scanning electron microscopy.

(II) For analysing the molecular mechanism of cerebral arteriogenesis, collateral tissue from the growing PCA was selectively isolated. Here, 24h post 3-VO 164 genes were detected to be significantly deregulated. Analysis of molecular annotations and networks associated with differentially expressed genes revealed that expression patterns contain gene transcripts predominantly involved in proliferation, inflammation, and migration. Early-phase cerebral arteriogenesis is characterized by protease inhibitor expression and showed that protease inhibitors TIMP-1 and kininogen are molecular markers of early-phase cerebral arteriogenesis.

(III) Functional relevance for kininogen-bradykinin signaling in arteriogenesis was analysed using the femoral artery ligation model in the mouse hind limb. 7 days post ligation arteriogenesis was significantly lower among B1-receptor (B1R) knockout (KO), B2R KO, and B1R/B2R double KO mice strains as compared to wild type animals.

In summary, this work characterizes morphological features and genomic profiles of growing collaterals in the brain and develops novel ideas for a therapeutic stimulation of arteriogenesis.

Keywords: Arteriogenesis, brain, gene expression, kininogen, bradykinin

Für Thilde Hillmeister "*Mümmi*" und meine Familie

Falls Gott die Welt geschaffen hat, war seine Hauptsorge sicher nicht,

sie so zu machen, dass wir sie verstehen können.

Albert Einstein

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Abbreviations

µm	mikrometer
3-VO	3-vessel occlusion
A2M	alpha-2 macroglobulin
ACA	anterior cerebral artery
AV shunt	arterial-venous shunt
B1 receptor	bradykinin receptor 1
B2 receptor	bradykinin receptor 2
d	day
DAVID	Data base, visualization, and Integrated Discovery
FGF	fibroblast growth factor
GM-CSF	granulocyte monocyte colony stimulating factor
GO	gene ontology
h	hour
HIF-1	Hypoxia-inducible factor - 1
ICAM-1	inter-cellular adhesion molecul 1
IPA	Ingenuity pathway analysis
KNT	kininogen
KO	knockout
LCN2	lipocalin 2
LDF	laser doppler flow
MCA	medial cerebral artery
MCP-1	monocyte chemoattractant protein
MMP	Matrix metalloproteinase
mRNA	messenger RNA
ng	nanogramm
NOS	nitric oxide synthase
NF-kB	nuclear factor-kappa B
PBS	phosphate buffered saline
PCA	posterior cerebral artery
PCAM	platelet-endothelial cell adhesion molecule
PCNA	proliferating cell nuclear antigen
qRT-PCR	quantitative real-time polymerase chain reaction
Rnase	Ribonuclease
RT	room temperature
s.c.	Subcutane
SAM	Significance analysis of microarrays
SEM	standard error of the mean
TGF	transforming growth factor
TIMP-1	tissue inhibitor of metalloproteinase-1
VEGF	vascular endothelial growth factor

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1. Introduction

The vascular system in mammals can been seen as one interconnected highly complex and organized organ system relevant for supplying oxygen and nutrients to all variants of tissue in the body (Risau, 1997). The function of the vascular system is of such importance that minor disorders can lead to severe disability and death. Cardiovascular disease is the leading cause of death in industrialized countries, among which stroke is the third most common cause of death and lasting disability. Still, therapeutic options are limited (Jones, et al., 2008; Lloyd-Jones, et al., 2009). Clinical studies have demonstrated the importance of an adequate hemodynamic compensation of cerebral blood flow to prevent brain ischemia in the context of atherosclerotic vascular diseases (Erdo and Buschmann, 2007). In order to develop a preventive concept against stroke, the understanding of the underlying vascular biology is of major concern.

Different forms of physiological vascular remodelling can be distinguished, of which arteriogenesis in particular has high clinical relevance. It was shown that arteriogenesis is the endogenous most efficient mechanism to compensate for an artery occlusion or stenosis (Carmeliet and Jain, 2000). Arteriogenesis denotes a proliferation and growth of pre-existent collateral vessels, classical anastomoses located e.g. in the myocardium, the lower extremities and the brain (Longland, 1953). Collateral arteries can dramatically increase their lumen by growth, bypassing the site of occlusion of a major artery and preventing imminent ischemia (concept of the biological bypass).

Experimental studies have provided evidence that growth of collateral arteries (arteriogenesis) can be enhanced therapeutically, which may reduce ischemic injury in endangered territories of the peripheral and cardiovascular system. In contrast to earlier descriptions of peripheral and myocardial arteriogenesis, corresponding results have long been missing for cerebral arteriogenesis. Recently, the three-vessel occlusion (3-VO) model in the rat was introduced to analyse cerebral arteriogenesis, which induces a compensatory growth of collateral arteries in the brain (Busch, et al., 2003). By occluding three of four extracranial arteries to the brain, one carotid artery and both vertebral arteries (3-vessel occlusion), a significant redistribution of blood flow is induced via the posterior cerebral artery (PCA), which showed a significant positive outward remodelling, increasing its diameter by 38 %. In addition, application of the pro-inflammatory granulocyte-macrophage colony-stimulating-factor (GM-CSF) in the

latter, same hypoperfusion model led to a further therapeutic increase in collateral growth (Heil, et al., 2000; Hossmann and Buschmann, 2005) and correlated with a significant reduction in experimentally induced stroke volume (Schneeloch, et al., 2004). This was the first evidence of therapeutic arteriogenesis in the brain (Love, 2003). In order to find novel therapeutic pharmaceutical compounds to treat arterial occlusive disease, increasing efforts are undertaken to unravel the molecular processes of adaptive arteriogenesis (Lee, et al., 2004),(Prior, et al., 2003), (Buschmann, et al., 2001).

The purpose of this study was to induce collateral growth in the rat brain by employing the 3-VO model and to subsequently investigate the early phase of gene expression within the selectively isolated proliferating collateral pathways. Hence, this work is dedicated to (I) characterizing the 3-VO model, (II) analysing the molecular biology of the mechanisms which drive early-phase cerebral arteriogenesis, and (III) identifying novel key factors of arteriogenesis. To our knowledge, this is the first study which analyses the molecular mechanisms of arteriogenesis in the brain.
The introduction briefly reviews the basic mechanisms of vascular remodelling in general (see section 1.1.) and will focus on the current knowledge about arteriogenesis (section 1.2.). Here, the introduction summarizes the morphological features of arteriogenesis (section 1.3.), and the methods and animal models in arteriogenesis research (section 1.4.). The introduction explains the 3-VO rat non-ischemic hypoperfusion model used in this study and shows its relevance for cerebral arteriogenesis research (section 1.5.). The genomic studies for arteriogenesis research are briefly summarized in section 1.6. Section 1.8 deals with the components and features of the kininogen system and bradykinin signaling, since molecular analysis of early-phase arteriogenesis highlight the prominent role of protease inhibitors, such as kininogen (part II). Functional relevance of kininogen-bradykinin signaling is shown in part III of this study.

1.1. Vascular Remodelling

The cardiovascular system plays a critical role in all vertebrates. Therefore, vascular development and maintenance consist of a highly organized series of events that requires the correct spatial and temporal expression of specific sets of genes. Three

different forms of vessel growth can be distinguished: vasculogenesis, angiogenesis, and arteriogenesis (Arras, et al., 1998; Buschmann and Schaper, 1999; Ito, et al., 1997).

Vasculogenesis describes the initial events in the development of the embryonic vasculature and comprises the *in situ* differentiation of mesodermal stem cells, termed angioblasts, into capillaries. The cardiovascular system is the first functional organ system to develop in the vertebrate embryo. Vasculogenesis leads to the arrangement of the first major intra-embryonic blood vessels (Risau, 1997), such as the dorsal aorta, the cardinal veins, and to the formation of the primary vascular plexus in the yolk sac. The basic network is guided by the unique structural and molecular features available in the embryo and is determined by genetic programming. With the onset of blood flow, these primary vessels have to be remodelled into arteries in order to develop a functional vascular circle. Organization of vascular development is increasingly taken over by feedback signals derived from vascular functions including blood flow and ischemia, which are the initial signals for arteriogenesis and angiogenesis. As a general rule, remodelling of the primary vascular plexus into a more mature vascular system as well as postnatal adaptation of the vascular system are thought to occur by angiogenesis and arteriogenesis.

Angiogenesis denominates the sprouting of endothelial cells leading to the development of capillary networks (Folkman, 2007) in normal physiological conditions (growth, exercise) and following cellular hypoxia as observed in pathologic conditions, such as systemic hypoxia, cancer, stroke, and other ischemic vascular disease. Pioneering work in the field of angiogenesis, especially with regard to the vascularization of tumors, has been performed by Folkman (Folkman, 1971). Tissue ischemia leads to the expression and activation of the transcription factor HIF-1. HIF-1 functions as a key regulator of oxygen homeostasis. Its expression leads to an increase of the transcription of several genes, including those encoding for NOS 1–3 and VEGF (Ryan, et al., 1998), (Carmeliet, 2005). In result, one of the first identifiable phenomena during tissue ischemia is vasodilation due to increased levels of NO and other not yet defined transmitters. Secondly, an increase in vascular leakage is observed due to the increased levels of VEGF (also known as vascular permeability factor). In fact, the occurrence of edema is a strong predictor of the angiogenic response. Apart from oedema, VEGF also induces, albeit moderately, endothelial cell proliferation. In summary, angiogenesis results in the de novo formation of capillaries in the hypoxic area, while the driving

force of angiogenesis is ischemia. Arteriogenesis, the main focus of this study, is, in contrast, independent of hypoxia and for ischemia.

1.2. Arteriogenesis

Arteriogenesis refers to a positive outward remodelling of pre-existing collateral arterial networks into larger calibre vessels which is a conserved mechanism in all vertebrates (Buschmann and Schaper, 1999). Collateral arteries are present in the newborn and adult organism. The presence of these pre-existent collateral connections was first reported in 1669 by Richard Lower at Oxford University. He observed very precisely the presence of pre-existent collateral connections between different vascular regions. Today, we know that pre-existent collateral arterioles are present in the peripheral, coronary, and cerebral circulation. Fulton et al. (Fulton, 1964) already recognized their function as alternative pathways for blood flow in case of flow deficiency, such as in case of an arterial disease.

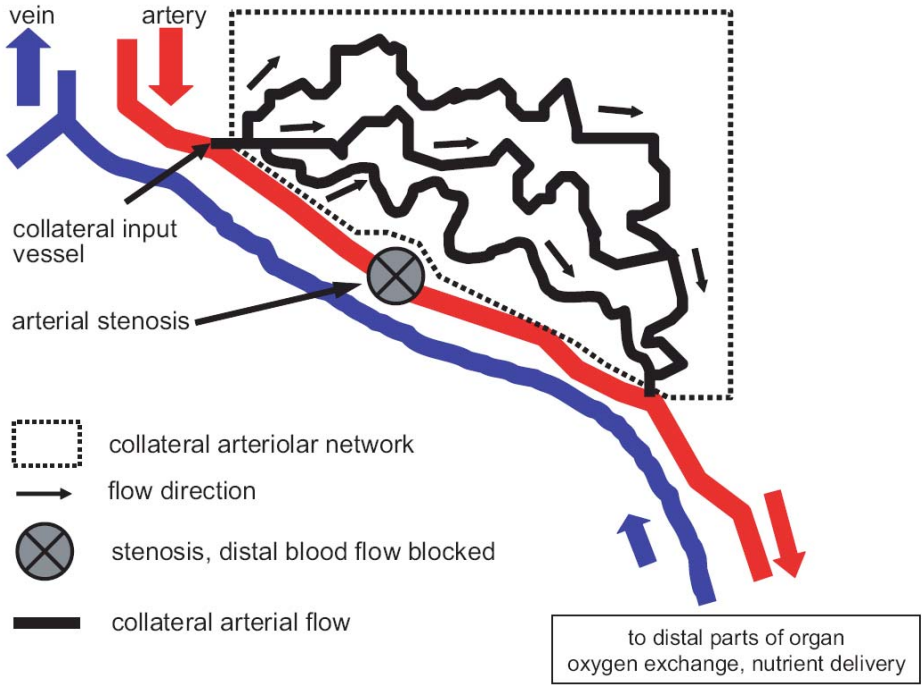

Figure 1. Principle of arteriogenesis

(Modified, F.LeNoble)

Arteriogenesis refers to the positive out-growth of collateral arterial networks following an arterial stenosis. Collateral arteries represent arteriolar anastomoses which interconnect different regions of major arteries. Therefore, in case of occlusion of a major artery, collateral arteries may function as a biological bypass which are capable of restoring blood flow to the ischemic area. In conclusion collateral artery growth determines clinical outcome.

An arterial disease is a condition that affects the arteries either by blocking or weakening blood vessels. Organs and other body structures may be damaged as a result of decreased or completely blocked blood flow (e.g. myocardial infarct or stroke), leading to a redistribution of flow to collaterals. Arteriogenesis constitutes an effective biological rescue mechanism against detrimental effects of arterial stenosis (Figure 1). In result, a small collateral artery grows into a bigger functional conductance artery, which circumvents sites of occlusion (Buschmann and Schaper, 2000): a biological bypass.

Hence, arteriogenesis is of high clinical relevance. However, in many cases adaptive arteriogenesis still falls short of complete hemodynamic compensation, since the rate of increase in collateralization falls below the increase of stenotic vessel occlusion. It is now recognized that stimulating arteriogenesis constitutes a potentially novel therapeutic option as a preventive concept against cardiovascular diseases being evaluated in the context of atherosclerotic vascular diseases (Ito, et al., 1997; Kalka, et al., 1999). Unfortunately, research that deals with therapeutic induction of vascular remodelling often does not correctly differentiate the forms of vascular growth. Still, frequently studies deal with counting de novo formed capillaries (angiogenesis) in the ischemic region which is not the sufficient method to gain qualified information about the pro-arteriogenicity of curtain compounds (Banai, et al., 1994). Arteriogenesis shares some features with angiogenesis, but the pathways leading to it are different, as are the final results: arteriogenesis is potentially able to fully replace an occluded artery, whereas angiogenesis cannot. In contrast to sprouting angiogenesis in the ischemic region, arteriogenesis deals with vessels outside the ischemic region in oxygen-rich tissue where a collateral system surrounds the site of occlusion (Pipp, et al., 2003). Increasing the number of capillaries within the ischemic region cannot significantly increase blood flow when its limiting structure lies upstream. Furthermore, according to the law of Hagen-Poiseuille, perfusion ν is mainly determined by the vessel radius r to the power of 4. Thus, only minimal changes in vessel diameter of a pre-existent arteriol lead to a big difference of the perfusion level (arteriogenesis). All other factors like viscosity η, pressure differences ΔP, and distance differences (vessel length) Δl as well as flow volume V are still proportional according to the equation.

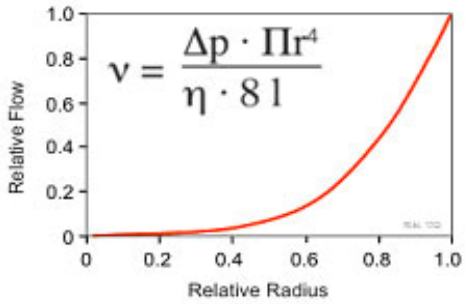

Figure 2. Law of Hagen -Posieuille

(Modified, Klabunde, R.E)

Equation clarifies the relationship between flow and vessel radius to the power of 4. Flow volume ν, viscosity η, pressure differences ΔP, distance differences (vessel length) Δl, radius r.

The relationship between flow and vessel radius to the fourth power is illustrated in Figure 2, and shows how very small increases in radius dramatically enhance flow.

In conclusion, newly formed small vessels cannot compensate for the loss of a main supplying arterial vessel (angiogenesis). This basic physiological principle has been shown in a number of clinical trials, where therapeutically induced angiogenesis has led to a significant increase in capillary density, but without significant beneficial effects on tissue perfusion (Storkebaum and Carmeliet, 2004; Wang, et al., 2005; Zadeh and Guha, 2003). In contrast, adaptive proliferation of collateral vessels (arteriogenesis) represents the most important physiological protection mechanism against arterial stenosis (Carmeliet, 2000).

For developing a potent therapeutic strategy to stimulate arteriogenesis, the genomic program and basic mechanisms of arteriogenesis need to be analysed. This will help to invent new medicinal therapies and lay the foundation for a strategy to therapeutically induce cerebral arteriogenesis as a preventive concept for stroke.

1.3. The Mechanisms of Arteriogenesis

Thoma et al (Thoma, 1893) found that vessels that carry a lot of blood flow enlarge, while those that carry little flow degenerate. Murray subsequently postulated that vessels adapt to flow in order to optimize the shear stress to which they are subjected (Murray, 1926). These studies have shown that flow can alter luminal dimensions of arterial segments. In fact, arteries are permanently exposed to hemodynamic forces because of the pulsatile nature of blood pressure and flow. Hence, the endothelium is constantly detecting different biomechanical forces, cyclic stretch and shear stress in

particular, and converts the latter stimuli into intra- and extracellular signals. Occlusion of a major artery leads to a significant increase in blood flow, resulting in high levels of shear. Shear force, not ischemia, plays the key role in adaptive phases of collateral artery growth, and was shown to be the driving force of arteriogenesis (Buschmann and Schaper, 2000; Hoefer, et al., 2004; Ito, et al., 1997). In fact, the overall impact of shear forces was shown by Schaper and colleagues, who induced an abrupt increase in shear stress by means of an arterial–venous (AV) shunt. An AV-shunt leads to a large and permanent pressure gradient (pressure drop across collateral arteries) and forces most of the collateral flow to empty into the vein. The result of shunting was a marked increase in collateral flow, which accelerated the speed of arteriogenesis (Eitenmuller, et al., 2006) (Figure 3).

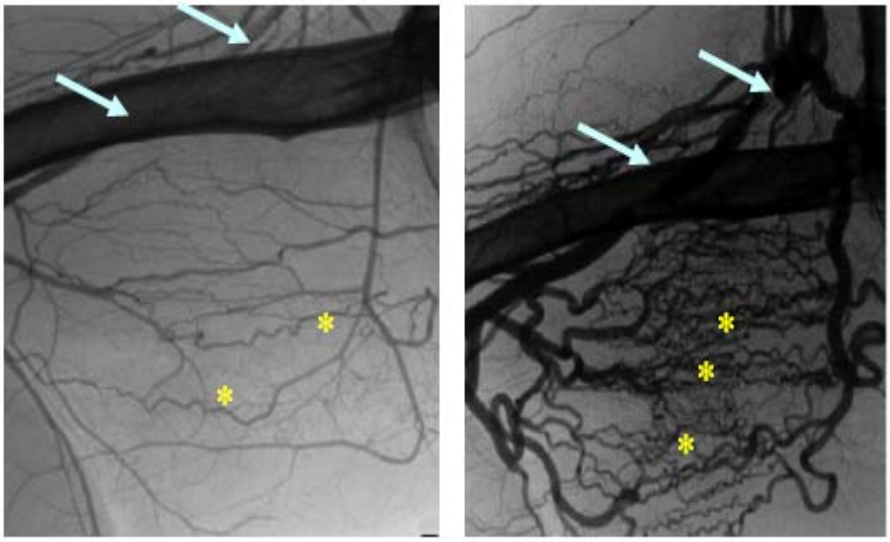

Figure 3. Therapeutic stimulation of arteriogenesis

(Modified, Eitenmüller, Circ Res. 2006)

Angiography of the rabbit hindlimb 3 weeks post femoral artery ligation. Arteriogenesis can be stimulated by increasing fluid shear stress or by application of pro-inflammatory cytokines. A: before stimuation, B: following stimulation. White arrows indicate site of occlusion. Stars show collateral vessel area.

Shear stress must be present for several hours before the endothelium becomes activated, which manifests itself in swelling of endothelial cells, proliferation, and edema (Schaper, 2004). Mechanoactivation of endothelial cells results in an upregulation of a number of genes which modulate multiple physiological processes: production of vasodilators (eNOS), growth factors (FGF) and cell adhesion proteins (ICAM, VCAM), mediation of inflammation by NF-kB, and expression of chemota-attractants (MCP-1) (Hoefer, et al., 2004).

The secreted pro-inflammatory cytokines attract monocytes, which then adhere to endothelial cell adhesion molecules, transmigrate, and accumulate in the perivascular tissue of proliferating collateral arteries (Deindl, et al., 2001; Hoefer, et al., 2005). After infiltration, the monocytes mature to macrophages, producing again numerous cytokines such as FGF, MCP-1, and TNF-α and degrading enzymes (MMP-2, MMP-9), thereby creating the controlled inflammation necessary to remodel an arteriole into an artery (Schaper, et al., 1967). In result, extracellular matrix is degraded. The surrounding tissue, consisting of smooth muscle cells, endothelial cells, and fibroblasts is stimulated and starts to proliferate. Collateral arterioles develop into large collateral arteries via a positive outward remodelling (Fabricius, et al., 1996; Gupta, et al., 1999; Ma, et al., ; Schneeloch, et al., 2004). This scenario is largely similar to the processes occurring during tissue injury and other inflammatory processes, and the factors involved are often identical (Hoefer, et al., 2004; Joussen, et al., 2002). These findings and the observation that paracrine effects invading monocytes are a key feature of arteriogenesis constitute the basis for the recent clinical and experimental interest (Bergmann, et al., 2006; Polverini, et al., 1977).

Therapeutic stimulation of arteriogenesis is possible e.g. through the exogenous application of MCP-1, via administration of monocytes (Hoefer, et al., 2004; Park, et al., 2008), or GM-CSF (Love, 2003) by prolonged monocyte survival. Infusion of MCP-1 and GM-CSF result in rapid enhancement of proliferation of pre-existing vessels after occlusion of a major coronary or peripheral artery and significant improvement of blood supply by stimulation of collateral growth (Arras, et al., 1998; Buschmann, et al., 2001; Ito, et al., 1997; Seiler, et al., 2001; Van Royen, et al., 2001).

In conclusion, interplay between vascular and cellular reactions to hemodynamic and molecular signals regulates the induction of arteriogenesis. First, flow induces expression of genes in endothelial cells, and second, paracrine inflammatory process which correlate with monocyte invasion into the perivascular space (Schaper, 2004).

It is well known that the architecture of arterioles and collateral growth differ slightly in the periphery and the heart. Arteriogenesis in the heart is initiated by monocyte invasion. These cells adhere initially to the endothelial surface and invade the intima. Later on the perivascular space becomes populated with monocytes, which supply space for the expanding collateral vessels by removing tissue that is in the way. In contrast neointima is not well developed in the vascular periphery, as it was shown in the mouse hind leg. Here, only a few monocytes adhere to the shear-stressed endothelium, and

possibly leaky post-capillary venules may enable their marked invasion of the perivascular space (Cai and Schaper, 2008).

These basic principles of arteriogenesis, as shown above, reflect our current knowledge in collateral artery biology and could repeatedly be confirmed in several *in vivo* studies for coronary and peripheral arteriogenesis. Yet, it is completely unknown what governs cerebral arteriogenesis due to the lack of an appropriate animal model in the brain.

1.4. Animal models in arteriogenesis research

Stimulation of collateral artery growth (arteriogenesis) provides a potential alternative option for the treatment of patients suffering from occlusive artery disease. Therefore, researchers have established several animal models to investigate basic mechanisms and pharmacological modulation of collateral artery growth: a coronary model (dog (Wolf, et al., 1998)) and several peripheral models (mouse (Scholz, et al., 2002)), in rat (Herzog, et al., 2002), rabbit (Ito, et al., 1997), or pig (Pipp, et al., 2004).
The choice of the correct animal model is important concerning the outcome, the specificity for one of the vascular remodelling processes (arteriogenesis, angiogenesis, vasculogenesis), and finally the clinical relevance. For an arteriogenesis model it is relevant to have an occlusion of a major artery resulting in hypoperfusion of the distal region, however, without forming a region of absolute ischemia, leading to severe tissue necrosis (development of an infarct areal) (Deindl and Schaper, 2005).

Certain physiological and morphological features have to be evaluated within each model in order to study arteriogenesis. First, an important tool in evaluating arteriogenesis is the measurement of perfusion by laser-Doppler technique (LDF) to determine collateral perfusion under different conditions, which is particularly used in small-animal species such as mice and rats (Heil, et al., 2002; Silvestre, et al., 2002). LDF assesses blood flow by detecting the flow velocity of corpuscular blood components (erythrocytes) through the reflected laser beam.
Second, growing collateral have to be identified and characterized. Histological staining for proliferation markers (PCNA and Ki-67) allows identification of collateral arteries in which an examination of the diameter or lumen area after maximal vasodilation can be performed. Furthermore, staining for the macrophage marker CD68 shows macrophage invasion, a common feature of arteriogenesis as described above.

Comparing vascular proliferation and macrophage invasion at different time points, however, gives strong hints of the outcome of the arteriogenic process.

Currently there are a number of efforts underway to develop new *in vitro* systems as alternative methods to analyse the basic mechanisms of arteriogenesis in artificial arteries. *In vitro* models are frequently used to understand flow dependent activation of endothelial cells under simulated flow conditions which help to understand the processes of collateral growth. However, this approach is still not sufficient to mimic the *in vivo* situation and analyse the basic mechanism of collateral growth, which still need to be performed in well-characterized arteriogenesis animal models.

Thanks to the rising use of transgenic and knockout models, increasing efforts are made to transfer the study of arteriogenesis into the mouse. Since the introduction of transgenic mouse strains into experimental research, their accessibility has helped to improve our understanding of the biological mechanisms of vascular growth. Therefore, the mouse model would provide a powerful tool to further illuminate basic mechanisms of arteriogenesis. This is especially important for the functional analysis of novel candidate genes for clinical relevance in transgenic animal models (Pipp, et al., 2003) (Scholz, et al., 2003; van Royen, et al., 2003).

In this study, morphological and transcriptional patterns for cerebral arteriogenesis were analysed in the rat brain 3-VO model (1.5.). Consequently, the mouse hind limb model of femoral artery occlusion was used to analyse the functional relevance of kinin signaling for arteriogenesis in bradykinin receptor KO mice (1.6.).

1.5. The 3-VO model of cerebral arteriogenesis

Analyzing collateral arteries in the brain is challenging due to the anatomical conditions. However, a multitude of extra- and intracranial collateral systems, such as the leptomeningeal anastomoses of Heubner, the anastomotic pathways via the ophthalmic artery, or the circle of Willis provide the chance of improving blood supply under conditions of slowly progressing vascular occlusion (Hossmann, 1993). Here, the circle of Willis, named after the English anatomist Thomas Willis, is of particular interest (Figure 4). It comprises the anterior, medial and posterior cerebral arteries (ACA, MCA, PCA), and the anastomoses, which connect them. The brain is supplied

by blood through four arteries: two vertebral arteries and two carotid arteries (Delank, 2006; Poeck, 2006), which all feed into the cerebral circulation via the circle of Willis.

Recently, the three vessel occlusion model (3-VO) model was established by Busch et al. in rat, for the first time allowing the investigation of cerebral arteriogenesis under controlled conditions (Busch, et al., 2003). Due to the small size of a mouse, the 3-VO model is yet only technically applicable in rats, since a controlled paravetebral occlusion of the vertebral arteries in mice is not feasible. Furthermore, the morphology of the cerebral arteries is very similar between humans and rats, including anomalies and structure of these vessels as well as their morphologic changes associated with cerebral vascular disease (Lee, 1995) (Figure 4).

3-VO describes a bilateral occlusion of the vertebral arteries in combination with unilateral carotid artery occlusion, which leads to cerebral hypoperfusion. The perfusion via the open right carotid artery accounts for an efficient cerebral perfusion, preventing neurological deficits.

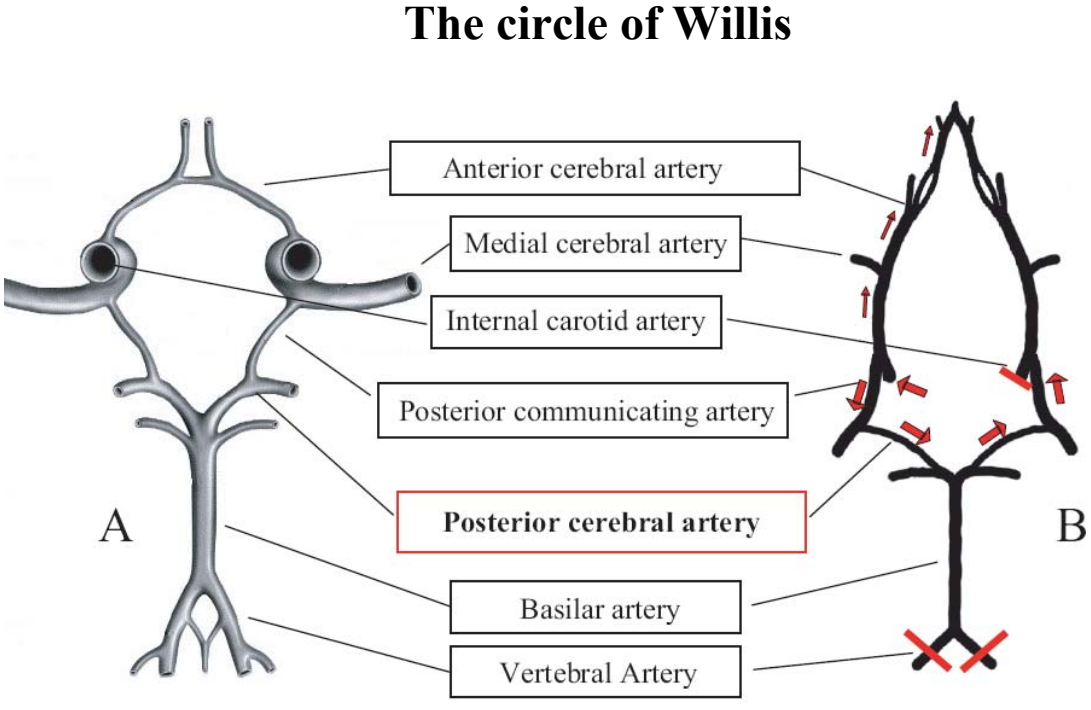

Figure 4. Circle of Willis. 3-vessel occlusion model in the rat brain.

A: Circle of Willis in man (Modified, Lee 2005), B: Circle of Willis in rat (Paxinos, 2004). Red barrs indicate occlusion of three vessels, one carotid, both vertebral arteries. Red arrows indicate redistribution of blood flow following 3-VO surgery. Post 3-VO blood may circulate into the brain via the carotid artery contralateral of the occlusion (left side) and is redistributed via the PCA and ACA region to the ipsilateral brain region (right side).

By occluding three of four extracranial arteries to the brain, a significant redistribution of blood flow is induced via the posterior cerebral artery (PCA) (Figure 4), which shows a significant positive outward remodelling, increasing its diameter by 37 % within 7 days (Hillmeister, et al., 2008).

Morphological studies show that shortly after occlusion a lasting reduction in cerebrovascular reactivity (CVR) occurs, but without histomorphologically detectable localized ischemic-necrotic tissue damage (Busch, et al., 2003). The animals do not show neurological deficits. For the first time a cerebral arteriogenesis model was designed, which allows the study of collateral growth in the brain under controlled conditions.

Furthermore, Buschmann et al. could show that the arteriogenesis in the 3-VO model is therapeutically inducible by the application of the pro-inflammatory cytokine granulocyte-macrophage colony stimulating factor (GM-CSF). In the same hypoperfusion model, application of GM-CSF led to a further therapeutic increase in collateral growth (Buschmann, et al., 2003; Hossmann and Buschmann, 2005) and correlated with a significant reduction in experimentally induced stroke volume (Schneeloch, et al., 2004). This was the first evidence for inducible therapeutic arteriogenesis in the brain (Love, 2003).

These results provide the basis for the planned studies of a new strategy of stroke prevention as well as the treatment of acute cerebrovascular insufficiency by therapeutic stimulation of arteriogenesis (Love, 2003). However, since treatment with GM-CSF is associated with a number of unspecific side effects, the logical consequence of the results at hand is to strive for a more detailed insight into molecular mechanisms to identify more specific therapeutic targets. Therefore, gene expression profiling studies shall give a direction.

This study aims at a comprehensive understanding of the genomic program activated during early-phase collateral vessel growth in a rat model of cerebral adaptive arteriogenesis (3-VO).

1.6. The Femoral artery occlusion model in mouse

Ligation of the femoral artery in the hind limb, leaving all branches intact and functional, is the prototype of a pure arteriogenesis model, because it does not lead to ischemia and hypoxia at the site of vessel growth (Hoefer, et al., 2006).

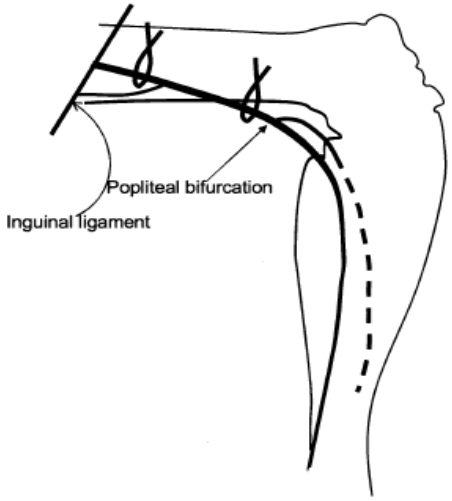

Figure 5. Femoral artery oclusion model (Modified, Lee, JACC 2004)

Schematic illustration of the femoral artery occlusion procedure performed in the mouse hind limb. In this arteriogenesis model femoral artery ligation is performed twice: distal to the inguinal ligament and proximal to the popliteral bifurcation.. Redistribution of blood flow results in collateral growth within the adductor muscle.

Arteriogenesis was studied first in rabbit (Deindl, et al., 2001). Rabbits display a rapid arteriogenic response and make for a highly successful model, regarding size and cost-efficiency. The femoral artery ligation model in the rabbit helped unraveling many processes of arteriogenesis in the periphery that can be stimulated (Figure 3) and inhibited, offering insights into mechanisms and potential therapeutic targets (Ito, et al., 1997). Because of the success of the femoral artery ligation model for inducing arteriogenesis, this model was also established in other rodent models, mouse and rat, as well as in pig (Voskuil, et al., 2003).

The femoral artery occlusion model in the mouse hind limb helps to directly compare quantitative data obtained from the use of transgenic animals or application of pharmacological inhibitors or stimulators of arteriogenesis. Therefore, studies dealing with arteriogenesis in different settings frequently analyse collateral growth in the femoral artery ligation model in mice. Quantification of collateral growth is performed by measuring the collateral conductance by microsphere perfusion. Collateral conductance directly describes arteriogenesis under situations of maximal vasodilation.

Conductance is the reciprocal value of the vascular resistance and describes increase in blood flow per increase in pressure.

$$\text{Conductance} = \frac{1}{R} = \frac{\dot{V}}{\Delta p}$$

Figure 6. Collateral Conductance

Collateral conductance is the reciprocal value of the vessel resitance. In situations of maximal vasodilation, controlled pressure determines blood flow in the collateral region.

Assessment of collateral-dependent flow using microsphere perfusion measurements remains the most precise experimental technique to quantify collateral growth and constitutes the gold standard in evaluating arteriogenesis (Schaper, 2004). However, the main disadvantages of this method are the direct and indirect costs, the necessity of taking tissue specimens for the quantification, and calculation of blood flow. Furthermore, collateral conductance in mice can be measured only by approximation, since ascertaining peripheral blood pressure is not feasible in mice. Still microsphere perfusion gives a capacity for collateral growth in mice which serves well to quantify arteriogenesis.

1.7. Molecular mechanisms

Whereas basic processes of arteriogenesis are understood, in which biomechanical endothelial activation and consecutive accumulations of monocytic cells are key initial events (section 1.3), the molecular mechanisms regulating this process are less defined. The development of collateral vessels is a tightly regulated and complex process requiring the action of multiple genes expressed in an appropriate time-dependent manner. In particular, it is important to know which genes are activated in the early-time processes of collateral growth in order to understand what the molecular mechanism initiating arteriogenesis is. Profiting from the general progress in molecular and cellular biology and the recent advances in microarray technology, the tools to perform comprehensive, quantitative comparisons at the transcriptional level of thousands of genes simultaneously are provided (King and Sinha, 2001), which help to make the mechanisms of arteriogenesis much clearer. The only relevant great scale genomic analysis so far was performed by a pioneer work of Lee et al. (Lee, et al., 2004), who studied collateral growth after femoral artery ligation in mice analysing 12.000 genes for differential expression using affymetrix microarrays. Herein he found

783 genes as deregulated and identified an early induction, mid-phase induction and late-phase induction of collateral growth.

In vitro studies identified genes expressed upon flow driven endothelial cell activation, such as KLF2 (Krüppel-like factor 2) (Dekker, et al., 2002), a VEGFR/PECAM complex (Tzima, et al., 2005), and the calcium channel TRPV4 (Taniguchi, et al., 2007; Vriens, et al., 2004). Furthermore, Schaper and collegues analysed gene expression in rapidly growing vessels in the rat hind limb by genome wide screening of transcripts using microarrays. Among the over 300 differentially expressed genes analysed, he found several of the known stress-responsive genes differentially expressed.
Similar studies for cerebral arteriogenesis were not performed. This is in particular significant, since Faber and colleagues documented for the first time that collateral density and vascular remodelling in the brain differ widely between mouse strains (Chalothorn, et al., 2007). For their study they analysed the leptomeningeal anastomoses of Heubner on the dorsal part of the brain, discovering significant differences in collateralization for BALB/c (lowest collateralization) and C57BL/6 (highest collateralization) mice strains. This indicates that collateralization is influenced by, as yet unknown, genetic factors. Here, first functional studies reveal that genes of chloride intracellular channel-4 (CLIC4) and VEGF-A (Chalothorn, et al., 2007; Clayton, et al., 2008) are determinant of native collateral formation in brain (Chalothorn, et al., 2009). Faber and colleges transferred their findings from collateral research of the brain to the mouse hind limb as well. As mentioned in section 1.6., arteriogenesis can be measured precisely in the femoral artery occlusion model and functional data can be compared to other studies. Thus, they found that collateralization in the skeletal muscle confirms the differences observed in the brain between mice strains (BALB/c and C57BL/6) (Helisch, et al., 2006), approving the relevance of CLIC4 and VEGF-A for arteriogenesis.

Different genetical factors determinate differences in collateral remodelling, which result in large differences in flow impairment after arterial occlusion. Thus, the density and diameter of native (pre-existing) collaterals in healthy tissues and their capacity to remodel in ischemia are major determinants of the severity of tissue injury in obstructive vascular disease (Helisch and Schaper, 2003; Schaper, 2009; Sherman, et al., 2006). Evidence suggests that native collateral conductance varies widely in healthy

individuals (Meier, et al., 2007; Wustmann, et al., 2003). Understanding the genomic program of early-phase cerebral arteriogenesis leading to collateral formation is of fundamental importance to develop a potential strategy to induce cerebral arteriogenesis therapeutically as a preventive concept for stroke.

1.8. The kininogen-bradykinin system

Molecular analysis identified kininogen as a molecular marker of arteriogenesis. This work is dedicated to analysing the functional role of the novel identified target genes, such as kininogen for arteriogenesis. Therefore, the kininogen-bradykinin system is briefly summarized.

Kininogen is cleaved by kallikrein which releases the nonapeptide bradykinin (Arg- Pro - Pro - Gly - Phe - Ser - Pro - Phe - Arg) and related kinins. The kinin system is a complex signaling network, that beside the precursor kininogens, consists of the proteolytic kallikrein enzymes, the Kinin peptides, and two G-protein-coupled receptors (GPCRs) termed the bradykinin receptor B1 and bradykinin receptors B2 (B1 and B2 receptor) that mediate the biological effects of kinin peptides (Figure 7) (Emanueli and Madeddu, 2001; Madeddu, et al., 2007).

There are two relevant kallikreins, which are encoded by distinct genes and differ in molecular weight, isoelectric point, amino acid sequence, immunogenicity, and susceptibility to inhibitors (Bhoola, et al., 1992). Tissue kallikrein is present in many tissues and various body fluids (Chao, et al., 2006; Chao, et al., 2001), whereas plasma kallikrein is almost exclusively found in circulating blood and is involved in the cascades of blood coagulation (Movat, 1979; Sainz, et al., 2007).

Furthermore, kallikrein was reported to increase MMP activity, which governs the homeostasis of extracellular matrix degradation for vascular remodelling (Figure 7) (Regoli and Barabe, 1980).

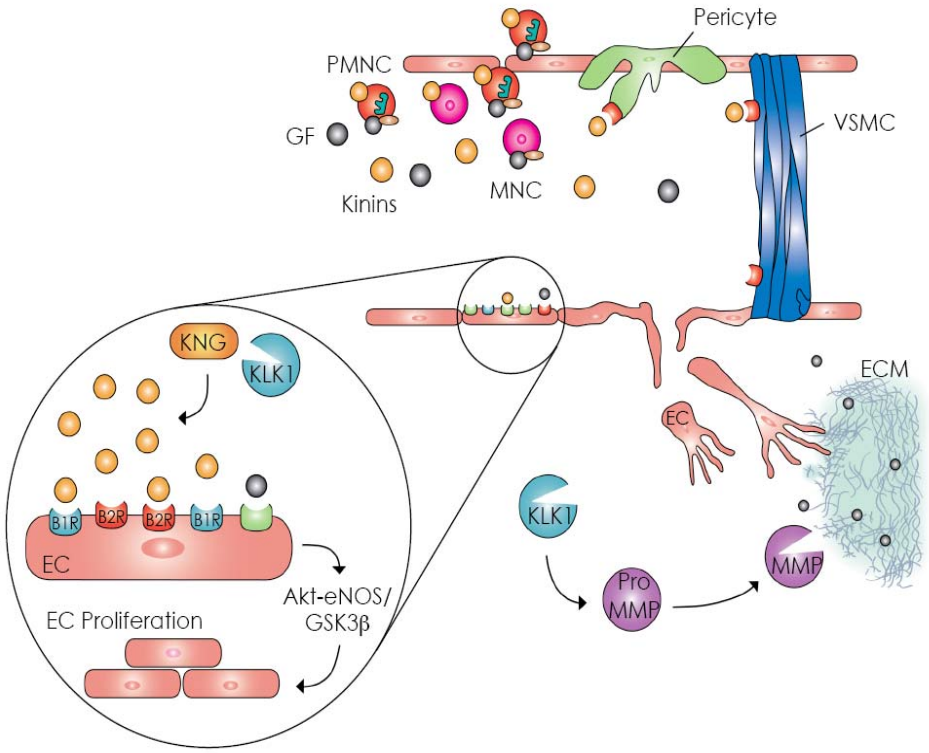

Figure 7. The kinin system in the vascular wall

(Modified, Stone, O 2009)

Kininogen (KNG) is processed by Kallikrein (KLK1) to cleave bradykinin and kallidin. Kinins signal via the constitutively expressed B2 receptor and the inducible B1 receptor. Stimulation of the B2 receptors on endothelial cells (EC) activates the Akt-eNOS/GSK3 pathways, which results in EC proliferation. Stimulation of the B1 receptor on ECs and leucocytes induce mono-(MNC) and poly- (PMNC) morphonuclear cells transmigration into the perivascular space. These processes induce expression of further kinins and growth factors (GF). B2 receptor signaling in vascular smooth muscle cells (VSMC) and pericytes enhances NO dependent vasodilation. Moreover, KLK 1 may increase MMP activity that may lead to breakdown of the extracellular matrix (ECM).

A single human kininogen gene encodes the production of two kininogen variants through alternative splicing: high-molecular-mass kininogen (HK; 88–120 kDa) and low-molecular-mass kininogen (LK; 50–68 kDa) (Chao, et al., 2006). Different kallikreins process the substrate kininogen, which liberates bradykinin and kallidin (also termed Lys-bradykinin). Bradykinin acts via the constitutively expressed bradykinin receptor 2 and kallidin via the inducible bradykinin receptor 1 (Regoli and Barabe, 1980). The kinin peptides are implicated in a wide range of biological phenomena, including pain, inflammation, vasodilation, increased vascular permeability, and natriuresis (Bader, 2009; Regoli and Barabe, 1980). Furthermore, the kinin system is reported to counteract the well-characterized renin-angiontensin system and vice versa. Both systems are the most important signaling pathways regulating vascular biology. Kinins are local hormones (autacoids), which are highly vasoactive compounds. Several

reports have demonstrated the presence of a kinin system in the vascular wall (Stewen, et al., 2004).

Kinins bind to the B1 and B2 receptor, which are found both on cultured smooth muscle cells as well as on endothelial cells (Figure 7). By signaling via the B2 receptor, bradykinin induces smooth muscle cell relaxation and vasodilation. Bradykinin signaling via the constitutively expressed B2 receptor on endothelial cells, results in EC proliferation, enhanced vascular permeability, and sprouting angiogenesis (Krankel and Madeddu, 2009). Stimulation of the B1 receptor leads to production of cytokines contributing to angiogenesis by paracine effects (Meneton, et al., 2001). Herein kinins share important features with VEGF (Ahluwalia and Perretti, 1999; Bhoola, et al., 1992). Mechanistically, kinin signaling is known to activate the Akt-eNOS/GSK3Beta pathway, increasing nitric oxide levels, and reducing reactive oxygen species formation (Yao, et al., 2008). Yet, a role for the kinin-system in arteriogenesis has not been described, which is remarkable since local increases in kinin level may attract mono- and polymorphonuclear cells, which themselves produce other growth factors - a major feature of arteriogenesis (Figure 7).

Pathophysiological models have shown a clinical relevance for the kinin system. It was shown that activation of kinin signaling protects against ischemic stroke and myocardial infarction (Xia, et al., 2006). Brown-Norway Katholiek rats, which lack secreted kininogen, showed a reduced capacity for new vessel formation (Ikeda, et al., 2004). Reduced angiogenesis was also observed by blocking the B1 and B2 receptor. Knockout mouse strains have been produced for each receptor type and have confirmed their predicted roles (Emanueli, et al., 2002; Meneton, et al., 2001). Homozygous mutated animals lacking the B1 gene or the B2 gene were shown to be viable and to present normal development (Pesquero and Bader, 1998). Hence, bradykinin receptor KO mice were used in this study to analyse the role of bradykinin signaling for collateral growth. Furthermore, for the first time the role of a double knock out mouse strain B1/B2 receptor was analysed in the context of vascular remodelling.

1.9. Research aims and objectives

Purpose of this study was to demonstrating how therapeutic strategies for stimulating arteriogenesis can be developed out of a comprehensive gene expression analysis. Therefore, this work aimed at three objectives: (I.) to characterize the 3-VO brain arteriogenesis model to analyse morphological features of cerebral collateral growth. (II.) In particular, this study aims at understanding the molecular mechanisms and transcription profile of early-phase arteriogenesis; to identify novel target genes, which govern collateral development in the brain. (III.) This study aimed at showing the functional relevance of a newly discovered molecular marker of the genomic profile of arteriogenesis (Figure 8).

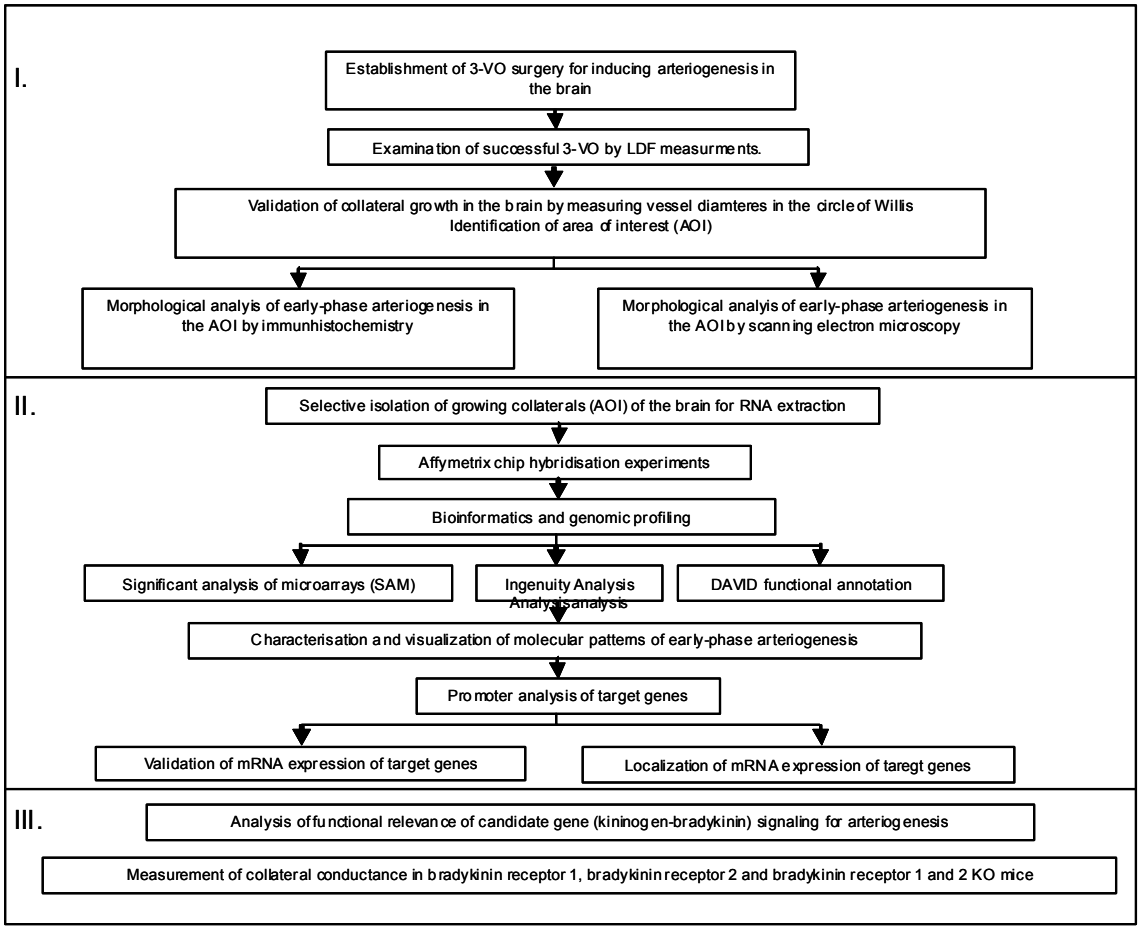

Figure 8. Flow chart of the study design

This work aimed at identifying, out of a comprehensive gene expression anlysis of early-phase cerebral collateral growth, candidate genes in order to develop noval concepts. Therefore this work is structured in three parts. The first part aimed at characterizing morphological features of early-phase cerebral arteriogenesis and identifying the area of interest for molecular analysis. Part two aimed at performing a global genom analysis to understand the molecular patterns of early-phase cerebral arteriogenesis. Candidate genes should be further analysed by biomolecular methods. Finally part three shall answer the question whether the newly identified target gene has functional relevance for arteriogenesis.

(I.) Cerebral arteriogenesis was characterized in the 3-VO model by blood flow and vessel diameter measurements. Here, a growing collateral artery (PCA) was identified as area of interest. Morphological features of the area of interest was analysed by approved methods of arteriogenesis research (immunhistochemical analysis), as well as newly established methods (electron microscopy of vessel intima).

(II.) To perform a comprehensive gene expression analysis tissue of the growing collateral was selectively isolated out of the rat brain, RNA extracted, and microarray hybridisation experiments were performed using the Affymetrix gene chip technology. Here, gene expression was analysed during early-phase of cerebral arteriogenesis to identify target genes, which may initiate arteriogenesis. Therefore, deregulated genes were clustered and annotated by several bioinformatics data bases (SAM, Igenuity, DAVID). Promoter regions of interesting target genes were scanned for regulatory elements, such as NF-kB binding sites and SSREs, and candidate genes (protease inhibitors). Protease inhibitors were identified as candidate genes and validated by qRT-PCR and mRNA expression localized by *in situ* hybridisation.

III. This study identified the protease inhibitor kininogen as target gene in early-phase cerebral arteriogenesis. For the purpose of vivo verification of data obtained in the molecular analysis, functional relevance of kininogen-bradykinin, signaling for arteriogenesis, was analysed using bradykinin receptor 1, bradykinin receptor 2, and bradykinin receptor 1/2 double KO mice. 3-VO model is yet not applicable to mice, for technical reasons (section 1.5.). Therefore, the technically available femoral artery occlusion model was used to assess the role of bradykinin signaling for arteriogenesis. This model is regularly used for the verification of data obtained in arteriogenesis research and allows precise assessment of arteriogenesis by measuring the collateral conductance.

2. Materials and Methods

2.1. Animal model

To analyze the morphological and molecular features of early-phase cerebral arteriogenesis, the 3-VO model in rat was used. Occlusion of the left carotid artery (CA) and both vertebral arteries was carried out as previously described in detail (Busch, et al., 2003) and is described in section 2.1.1. For analysing the functional relevance of the kinin-system using Bradykinin receptor KO mice, the femoral artery ligation model was used, since the 3-VO model is yet not applicable in mice. Furthermore, arteriogenesis was assessed in the hind limb by microsphere perfusion, which is the gold standard for measuring collateral growth. The femoral artery ligation model is described in section 2.1.2.

2.1.1. 3-VO surgery

The non-ischemic brain hypoperfusion 3-vessel occlusion model (3-VO) was used to induce adaptive arteriogenesis in the brain without detectable neurological damage (immunohistological evaluation), as described by described in detail by Busch et al. and Schneeloch et al (Busch, et al., 2003; Schneeloch, et al., 2004). Experiments were carried out in male Sprague-Dawley rats (300-350 g, Harlan-Winkelmann, Borchen, Germany) in accordance with the German Law for the Protection of Animals and the *National Institute of Health Guidelines for Care and Use of Laboratory Animals* (license for animal testing G 0360/05). Animals were housed under diurnal lighting conditions and allowed access to food and water ad libitum. Anesthesia was induced by inhalation of 2 % to 4 % isoflurane and maintained with 2 % isoflurane in oxygen. Analgesia was achieved by Buprenorphine s.c., 0.1 mg/kg intraoperatively, and 0.05 mg/kg s.c. BID for 2d after surgery. Vascular occlusions were carried out by electrocoagulation of both vertebral arteries, using a paravertebral access (Pulsinelli, et al., 1983), followed by left common carotid artery ligation. Successful 3-VO surgery was measured by laser Doppler flowmetry. At the end of experiments, animals were sacrificed, and the vessels of the circle of Willis were taken for RNA extraction or perfused with either ink-stained 37°C warm latex (via the left CA) for visualization of the cerebrovascular anatomy or with 4 % paraformaldehyde / PBS (via the left ventricle) for histological evaluation.

2.1.2. Laser Doppler flowmetry

To ensure cerebral hypoperfusion, CBF (Cerebral blood flow) was measured by transcranial laser Doppler flowmetry (LDF, PeriSoft, PeriMed, Järfälla, Sweden). Continuous laser Doppler flowmetry of both hemispheres was performed throughout the whole experiment. The skull above the frontoparietal cortex was exposed, and a laser probe was placed directly onto the skull bone. The LDF device was kept in an identical position before and after 3-VO. Changes of laser Doppler flow values are expressed as percentage of mean baseline values.

2.1.3. Visualization of cerebral angioarchitecture

Cerebrovascular anatomy was studied after maximal vasodilation by a modification of the postmortem latex perfusion method of Maeda et al (Maeda, et al., 1998). External PCA diameter was measured with a stereozoom microscope (Leica MZ6) equipped with a calibrated eyepiece micrometer. Data sets are presented as mean ± standard error of the mean. Diameter changes were analyzed for statistical significance by an unpaired Student's *t*-test. Statistical significance was assumed for $P < 0.05$.

2.2. Femoral artery ligation

Bradykinin receptor 1 KO (n=10), bradykinin receptor 2 KO (Jackson Laboratory, Bar Harbor, Maine) (n=10), Bradykinin receptor 1+2 dobble KO mice (n=10), and wild type mice (n=10) underwent unilateral femoral artery ligation. (Licence for animal testing TVV G 0108/08). All mice were male and between 12 and 14 weeks of age and had a C57BL/6 background. The right femoral artery was ligated immediately distal to the inguinal ligament. Because collateral arteries develop from preexisting arteriolar connections spanning from the profunda femoris and circumflexa femoris to the genualis and saphena parva arteries, the femoral artery was not excised to leave these vessels intact. Wounds were closed and animals recovered. After the surgical procedure, the animals were housed in groups of 5 with free access to water and chow and were allowed to move freely. There were no signs of any gross impairment or necrosis.

2.2.1. Collateral Conductance measurement

Seven days after ligation, mice were anesthetized for microsphere-based flow calculations. To ensure perfusion of both hind limbs and optimal distribution of the

fluorescent microspheres, the abdominal aorta was cannulated with a polyethylene catheter (inner diameter, 0.58 mm; outer diameter, 0.96 mm). After stabilization of systemic pressure, both legs were perfused at 4 different pressure levels (70, 80, 90, and 100 mm Hg, respectively) with saline and adenosine (1.0 mg/kg per min, Sigma) to achieve maximal vasodilation. At each pressure level, microspheres with a different fluorescent dye (red, blue-green, orange, or yellow-green; diameter, 15 _m; Molecular Probes) were thoroughly mixed and injected into the perfusion system. The hind limb muscles were dissected from the leg, weighed, homogenized, and digested with SDS/proteinase solution for FACS analysis and microsphere counting.

2.2.2. Microsphere counts

The following muscles were dissected from the leg: quadriceps, adductor longus, adductor magnus, gastrocnemius, soleus, plantaris and peroneal muscles. Each muscle was divided into three consecutive samples (0.5g) from the proximal to the distal end. Each muscle sample was weighted and samples were then homogenized and placed loosely in 15ml tubes (Becton Dickinson, Lincoln Park, NJ). To each of the tissue samples, the following was added: 3 ml of a proteinase/SDS solution [SDS stock solution: 1% SDS, 0.5 % sodium azide (both Sigma Chemical Company, St. Louis, MO) and 0.8 % Tween-80 (Fisher Scientific, Fair lawn, NJ) in 50 mM pH 8 Tris buffer (Sigma Chemical Company, St. Louis, MO)] and 1 mg/ml proteinase K (Boehringer Mannheim Corp.). Blue microspheres (4000/ml, diameter: 15 mm; Molecular Probes, Eugene, Oregon, USA) were used as an internal standard. Each tube was capped and secured in a shaking water bath at 50°C for 24 h. All samples were then centrifuged at 1000*g* for 30 min; the supernatant was pipetted off and the pellet was resuspended in 1 ml CellWash (Becton Dickinson, Lincoln Park, NJ). Directly before FACS analysis, the probes were rigorously shaken. For microsphere counting, a flow-cytometer (FACSCalibur) equipped with a second laser and a detector for a fourth fluorescence was used. After FACS analysis, each single microsphere was classified and counted with a computerized analysis system (Becton Dickinson, Lincoln Park, NJ). Hind limb collateralization was estimated by normalizing the number of microspheres in the sample with microspheres of a reference samples. The unligated left hind limb was processed in the same manner, as described above, defined as 100 % and served as the internal validation. Values generated represent a value for the perfusion reserve of the hind limb, which estimates collateralization and arteriogenesis. Data sets are presented as mean ± standard error of the mean. Diameter changes were analyzed for statistical

significance by a Bonferroni *t*-test. Statistical significance was assumed for $P < 0.05$, and <0.01.

2.3. Histological analysis

For immunhistochemical analysis of the morphological features of cerebral arteriogenesis rats were submitted to occlusion of the left carotid artery (CA) and both vertebral arteries (3-VO) and enrolled into the study as follows: Control group (n=6); 24h post 3-VO (n=6); 24h post sham (n=6); 3d post 3-VO (n=6); 3d post sham (n=6). Information regarding the used antibodies is provided in Table 4 section 2.8.2. Images were obtained using a Leica DM-R Microscope.

2.3.1. Embedding, fixation and pretreatment of paraffin-embedded sections

Brain tissue was cut into sagittal or coronal parts and submerged in fixative (0.01M PBS; 4 % paraformaldehyde) for 16 h at 4 °C overnight and subsequently washed in PBS at room temperature. Fixed tissues were dehydrated and paraffinized (Paraffin 9) in an embedding machine (Shanon Citadel 1000, Thermo). The embedding program included the following steps: 70 % ethanol for 1h, 96% ethanol for 1h, 100% ethanol for three changes (1h each), acetone for 30min, and paraffin wax for three changes (2x 1 h and 1x over night). Tissues were then embedded into paraffin blocks and cut on a microtome (Microm) in 5 µm sections, mounted onto slides, dried for 30 min and incubated at 60 °C for 1h. Paraffin sections were de-paraffinized for 5 min each in xylene (3 changes), 100 % ethanol, 96 % ethanol, 80 % ethanol, 70 % ethanol, and water.

2.3.2. Immunoflourescence detection

For epitope retrieval slides were boild in citrate buffer (10mM, Tween 20, pH 6.0) for 20 min in a microwave, cooled down to room temperature for 40 min, rinsed in dH2O, and stored in PBS. The slides were rinsed in washing buffer (PBS, 0.05 % Tween 20, pH 7.2) and blocked with serum blocking solution (1 % serum, 1 % BSA, 0.1 % Triton X-100, 0.05 % Tween 20) for 30 min at room temperature. The used serum was derived from the same species as the secondary antibody (usually goat).

Then, the sections were covered with the primary antibody diluted in PBS including 1% BSA for 1 hour at room temperature, and rinsed three times for 2 min in PBS. The sections were incubated with fluorescently labeled secondary antibodies (Cy3) diluted

1:200 in PBS for 30 minutes at room temperature and rinsed three times for 2 min in washing buffer. Cell nuclei were stained using Hoechst 33342 (1:1000 PBS, Molecular Probes Inc., Eugene, Oregon, USA). Slides were coverslipped with Flouromount-G (Southern Biotech). Twelve sections of each animal were analysed in a blinded approach by three independent investigators.

2.3.3. Immunoenzyme (HRP) method

Epitope retrieval, washing and blocking was performed as described above. Endogenous peroxidase activity was blocked by incubating paraffin sections in 3% H_2O_2 in PBS. The sections were covered with the primary antibody diluted in PBS for 1 hour at room temperature or overnight at 4 °C and rinsed three times for 2 min in washing buffer. The sections were then incubated with a biotinylated secondary antibody (1:200, Vector) diluted in PBS for 30 minutes at room temperature and rinsed three times for 2 min in washing buffer. The biotinylated secondary antibody was detected with a complex of streptavidin conjugated to horseradish peroxidase (Strept ABComplex/ HRP, Dako) for 30 min at room temperature. To visualize conjugated horseradish peroxidase, the sections were covered with AEC solution (Dako) and counterstained in Mayers haematoxylin (Sigma). Finally, sections were coverslipped in aqueous mounting medium (Aquatex, Merk).

2.4. Scanning electron microscopy (SEM)

For scanning electron microscopy, male adult (300-400g) Sprague-Dawley rats (n=16) were sacrificed 24h or 3d after 3-VO or sham operation.

Animals were deeply anaesthetized by intraperitoneal injections of 45% ketamine (100 mg⁄ml), 35% xylazine (10 mg⁄ml) and 20% saline at a dose of 0.32 ml ⁄ 100 g of body weight. They were given 200 IU heparin i.p. and perfused transaortically with pre-warmed plasma substitute (Deltadex 60, DeltaSelect, Pfullingen, Germany, for 10 seconds at 38°C), followed by perfusion fixation with 1.5% formaldehyde, 2.5% glutaraldehyde and 0.01% methylene blue (as label) in 0.1 M phosphate buffer, pH 7.4. After 25 minutes, fixation was terminated by perfusion with 5% sucrose in 0.1 M phosphate buffer, pH 7.4, for additional 5 minutes.

Posterior cerebral arteries were isolated and dehydrated in ethanol concentrations of 30% (2h), 50% (2h), and 75% (O/N). Samples were opened lengthwise, treated with OsO_4 (4%, 2 hrs), critical-point dried (CPD 30, Bal-Tec), sputtered with ionized gold in

a high-pressure argon atmosphere (CDC40), and viewed under a scanning electron microscope (Quanta 200, FEI, Kassel).

2.5. Gene expression profiling

2.5.1. Area of interest and selective tissue isolation

For RNA extraction, animals were submitted likewise to the 3-VO surgical procedure. 24 h and 3 days after occlusion, the ipsilateral and contralateral part of the PCA within the circle of Willis and the posterior communicating artery were isolated and considered as our area of interest to study cerebral arteriogenesis, as shown in Figure 13. RNA isolation for gene expression analysis was performed for five treatment groups: 24h post 3-VO; 24h post sham, 3d post 3-VO, 3d post sham, and untreated control group. For each treatment group, PCA of 24 animals were isolated and split into three independent pools of vessels. Each pool consisted of PCA from 8 animals. Three affymetrix cDNA microarrays were hybridized (one array for each pool) for each treatment group (24h post 3-VO (n=3), 24h post sham (n=3), 3d post 3-VO (n=3), 3d post sham (n=3) and control (n=3).

Table 1. Gene chip hybridisation experiment study design

24 h post 3-VO	**3 days post 3-VO**	**24 h post sham**	**3 days post sham**	**untreated**
n = Pool of PCA from 8 animals	n = Pool of PCA from 8 animals	n = Pool of PCA from 8 animals	n = Pool of PCA from 8 animals	n = Pool of PCA from 8 animals
n = 3	**n = 3**	**n = 3**	**n = 3**	**n = 3**

2.5.2. RNA isolation and quantification

Total RNA was isolated using the RNeasy kit (Qiagen). The quantity and quality of extracted RNA was further assessed using the RNA 6000 Nano LabChips Kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's instructions.

2.5.3. Microarray hybridization

For each array, total RNA (see above) was processed. Biotinylatted cRNA was hybridized to affymetrix chips by the Institute for Functional Genomics (Charité, Berlin, Germany). Gene expression was analyzed using the commercially available Rat 230 A (Affymetrix) Gene Chip containing 15866 probe sets. Hybridization, washing, antibody amplification, staining, and scanning of arrays were performed according to the

Affymetrix technical manual. Arrays were scanned using the GeneChip System (Hewlett-Packard, Santa Cruz, CA) and raw data were processed using GCOS and normalized to a global intensity of 500.

2.5.4. Microarray data analysis

All microarray data were analysed using significance analysis of microarrays [SAM (1.21)] with logarithmic transformation to identify differential gene expression after 3-VO (false discovery rate <10%). The fold change in gene expression levels of each gene was calculated as relative to data from sham-operated animals. Significantly deregulated genes were further analysed using Ingenuity Pathways Analysis IPA 3.0, which identifies physical, transcriptional, or enzymatic interaction networks.

Ingenuity Pathways Analysis is a web-based software application containing most literature knowledge of biologic interactions between gene products (http://www.ingenuity.com/products/pathways_analysis.html). In this study, the gene expression profile is presented by the networks generated by Ingenuity: displayed in list form the significantly deregulated genes together with the according affymetrix gene identification number and fold-change expression values. Furthermore, expression profiles of those genes were visualized and presented using Gene Math 1.5.

In order to understand molecular and biological relations of candidate genes, significantly deregulated genes were functionally annotated using the web-based **D**atabase for **A**nnotation, **V**isualization, and **I**ntegrated **D**iscovery (DAVID) (Dennis, et al., 2003).

2.5.5. Target gene promoter analysis

For 53 genes, DNA sequences from the Ensembl database (ENSEMBLREF) were collected, here, a region 5000 bp before and 1000 bp after the transcription start site was defined as promoter region and analysed for: (a) putative NF-kB binding site and (b) shear stress response elements (SSRE). Both strands were scanned with (a) the consensus element KGGRAANTCCC (Sen and Baltimore, 1986) and (b) the potential SSRE sites GAGACC and ACCKAGACCAG (Houston, et al., 1999), allowing one mismatch and using the program *fuzznuc* of the EMBOSS package (EMBOSSREF). Here R stands for A or G, K for T or G.

2.6. Validation of target genes

2.6.1. cDNA synthesis

500 ng RNA was then reversely transcribed to first-strand cDNA using random hexamer primers in 20 µl reactions, ribonuclease inhibitor (0.5 U/µl) and SuperScript II reverse transcriptase (RT) provided in SuperScript First-Strand synthesis kit (Invitrogen, Carlsbad, California) following the manufacturer's protocol.

2.6.2. Quantitative real time PCR

Amplification was carried out in the ABI Prism 7000 thermocycler (Applied Biosystems) with SYBR Green dye (Applied Biosystems) and 2 ng cDNA as template in 25 µl reactions. No-template controls and non-enzyme controls were included. Products were analysed by gel electrophoresis and inspection of dissociation curves. Quantitative real time PCR (qRT-PCR) was performed using gene-specific primers, which were designed using the Primer 3 software (Rozen and Skaletsky, 2000), and sequences are provided in Table 2 and Table 3. Gene expression data were normalized against HPRT, B-Actin, and GAPDH. The PCR profile used for qRT-PCR was 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 15s, and 30 s for 72°C. The cycle number, at which the emission intensity of the sample rises above the baseline, was referred to as Ct (threshold cycle) and was proportional to target concentration. Real time PCR data are given as averages of three independent experiments, while each experiment was done in triplicate. Quantification was performed using single reactions and was analysed by the DART-PCR method (Version 1.0) (Peirson, et al., 2003) or GED-standard curve method as described by *Schefe et al.* (Schefe, et al., 2006). Mean PCR efficiency of gene-specific reactions was used for data analysis. Data are expressed as the means ± standard error of the mean. Differences were evaluated with the Mann-Whitney-U-Test; p-values <0.05 were considered significant.

2.7. *In situ* hybridization

2.7.1. Cloning

Probes were cloned using the pGEM-T construct (Promega), which carry an ampicillin resitance site. Tables of primers may be found in section 2.8.1. Chemically competent

DH5α E.coli cells (Invitrogen) were used for the propagation of standard vectors and routine subcloning. PCR for the cloning approaches was performed with High Fidality Taq DNA polymerase (Roche, PCR system).

2.7.2. Agarose gel electrophoresis

DNA molecules were separated by gel electrophoresis in gels containing 1-1.5% agarose. Standard electrophoresis grade agarose (Invitrogen) was used for analytical gels. The electrophoresis was performed in a custom system (Bächler Feintech) at 50 V for 20-40 min. The buffer system was TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.3) in case of analytical gels and TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) for cloning approaches. Ethidium bromide (50 µg/l) was included in the gel to visualize DNA bands by UV light (Syngene).

2.7.3. Plasmid isolation and gel extraction

For plasmid isolations, 3-4 ml 2YT-medium, including proper antibiotics (Ampicillin) was inoculated with one colony and cultured overnight at 37 °C while shaking (225 rpm). Cells grown in 3-4 ml overnight culture were pelleted and plasmids were isolated by QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's protocol and eluted in 30 µl H_2O. If a larger amount of plasmid DNA was desired, 100-200 µl of the overnight culture was used to inoculate 100 ml 2YT medium. On the next day, plasmids were isolated by QIAGEN Plasmid Maxi Kit (Qiagen) according to the instruction manual. Plasmids were eluted in 200 µl dH2O and stored at - 20 °C.

PCR products were purified by QIAquick® PCR Purification Kit (Qiagen) and eluted in 30 µl dH_2O. Restricted DNA fragments of linearized vectors were separated by gel electrophoresis, cut from the gel, extracted using QIAquick® Gel Extraction Kit (Qiagen), and eluted in 30 µl dH_2O.

2.7.4. Ligation and transformation of chemically competent bacteria

DNA inserts and linearized vectors were ligated by T4 DNA ligase (Invitrogen) according to manufacturer's protocol. Ligations were incubated overnight at 16 °C and diluted 5-fold in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) before adding to competent cells.

Chemically competent DH5α E.coli cells (40 µl) were thawed on ice and mixed with 10-100 nl plasmid. The reaction was pre-chilled for 5-30min, heat-shocked at 42°C for 45 sec, and subsequently incubated for 2min on ice. The cells were resuspended in 1 ml

2YT-medium, incubated at 37 °C for 30min while shaking, and plated onto selection agar plates (Ampicillin).

2.7.5. Sequencing and *in vitro* transcription

Successful cloning was checked by DNA sequencing (Genome Center, Humboldt-University, Berlin). For probe generation, 10 µg of each construct were digested by NotI or NcoI. Enzymatic reaction was purified by the QIAquick® PCR Purification Kit (Qiagen). Finally, probes were amplified by in-vitro transcription using T7 polymerase (sense probe) or SP6 polymerase (antisense probe).

2.7.6. *In situ* hybridization

In situ hybridization (ISH) was performed on 5µm paraffin embedded tissue sectins after postfixing them in 4% PFA in phosphate buffer (pH 7.4; 30min), thereafter rinsing with phosphate buffer and digesting with Proteinase K (10µg/ml) in TRIS-EDTA buffer (pH 8.0;10min). Sections were hybridized for 18h at 65°C using digoxigenin-labeled probes. Immunostaining for digoxigenin (Dig) was performed with an anti-Dig antibody conjugated to alkaline phosphatase (Roche, Indianapolis, USA) using NBT/X-Phosphate as a substrate (blue staining). For TIMP-1, a probe of 336 bp, and for KNT a probe of 496 bp was generated. Primers are shown in Table 2 (Section 2.8.1.).

2.8. Assessment of systemic inflammation markers – White blood cell count serum amyloid alpha measurement

To differentiate local cell invasion from systemic post-operative acute phase effects, typical markers of a post-surgical acute phase reaction were assessed in the bloodstream of all 3-VO, sham, and control animals. Blood samples were taken from each animal group and analysed by the Institute for Veterinary Medicine Diagnostics (VMDI, Berlin, Germany) for differential WBC count and serum amyloid A levels using flow cytometry, and solid phase ELISA with biotinylated monoclonal anti-SAA antibody (Tridelta, Ireland), respectively. Analyses were performed in a blinded fashion and statistical significance analysed by Student's *t*-test. Statistical significance was assumed for $P < 0.05$.

2.9. Material

2.9.1. Primers

All primers were obtained from Invitrogen Custom primers in a 25 nmol scale (HPSF purification) and resuspended in H_2O to 100 pmol/µl (100 mM) stock concentration. Forward and reverse primers were diluted with RNase/DNase free water (Gibco) to a working concentration of 10 µM each.

Table 2. Primer List 1

Primer for qRT-PCR	**Gene Name**	**Sequenz 5´ ⟶ 3´**
Candidate Genes		
rtKNT1-forward1	Kininogen	CACAGGTGGTTGCTGGAGTA
rtKNT1-reverse1	Kininogen	TCACAATCCGCTGTCAGTTC
rtTIMP1-forward1	Tissue Inhibitore of Metalloproteinase 1	GCACAGTGTTTCCCTGTTCA
rtTIMP1-reverse1	Tissue Inhibitore of Metalloproteinase 1	ATCGCTCTGGTAGCCCTTCT
rtLCN2-forward1	Lipocalin 2	CCCTGTACGGAAGAACCAAG
rtLCN2-reverse1	Lipocalin 2	GGTGGGAACAGAGAAAACGA
rtA2MG-forward1	Alpha-2-Macroglobulin	TTCTCTTCCACCCAGGACAC
rtA2MG-reverse1	Alpha-2-Macroglobulin	CGAAGAATGGATGGTCACCT
Housekeeping Genes		
rtGAPDH-forward1	GAPDH Glycerin aldehyd-3-phosphat dehydrogenase	CATCAACGACCCATTCATTG
rtGAPDH-reverse1	GAPDH Glycerin aldehyd-3-phosphat dehydrogenase	TTCCCATTCTCAGCCTTGAC
rtGAPDH-forward2	GAPDH Glycerin aldehyd-3-phosphat dehydrogenase	TGCACCACCAACTGCTTA
rtGAPDH-reverse2	GAPDH Glycerin aldehyd-3-phosphat dehydrogenase	GGATGCAGGGATGATGTTCT
rtHPRT-forward1	HPRT Hypoxanthine-guanine phosphoribosyl transferase	CTCATGGACTGATTATGGACAGGAC
rtHPRT-reverse1	HPRT Hypoxanthine-guanine phosphoribosyl transferase	GCAGGTCAGCAAAGAACTTATAGCC
rtHPRT-forward2	HPRT Hypoxanthine-guanine phosphoribosyl transferase	GCAGACTTTGCTTTCCTTGG
rtHPRT-reverse2	HPRT Hypoxanthine-guanine phosphoribosyl transferase	GGCCTGTATCCAACACTTC

Primer forward in situ hybridisation	**Gene Name**	**Sequenz 5´ ⟶ 3´´**
ISH-KNT-forward1	Kininogen	AACATCACAGGTGGTTGCTG
ISH-KNT-forward3	Kininogen	AAGAGTGCCCACTCACAGGT
ISH-KNT1-reverse1/3	Kininogen	CCTTTGAGAGTCTGCCCTTG
ISH-TIMP1-forward2	Tissue Inhibitore of Metalloproteinase 1	TCCCCAGAAATCATCGAGAC
ISH-TIMP1-forward3	Tissue Inhibitore of Metalloproteinase 1	CTTTGCATCTCTGGCCTCTG
ISH-TIMP1-reverse2/3	Tissue Inhibitore of Metalloproteinase 1	TGAACAGGGAAACACTGTGC
ISH-NGAL-forward3	Lipocalin 2	CTGGGTGTCCTGTGTCTGG
ISH-NGAL-reverse3	Lipocalin 2	CTTGGTTCTTCCGTACAGGG
ISH-A2MG-forward1	Alpha-2-Macroglobulin	CATTTGCCCTTGCTGGTAAC
ISH-A2MG-forward2	Alpha-2-Macroglobulin	TCAGCAGCAGAAGGACAATG
ISH-A2MG-reverse12	Alpha-2-Macroglobulin	GTGTCCTGGGTGGAAGAGAA

Sequences of oligonucleotides (candidate genes) used as forward or reverse primers for qRT-PCR and *in situ* hybridisation.

Table 3. Primer List 2

Other genes tested	Gene Name	Sequenz 5´ ⟶ 3´
rt-tKNG-for1	t_Kininogen	GCCTCCAGGATTTTCACCTT
rt-tKNG-rev1	t_Kininogen	CCTTTGAGAGTCTGCCCTTG
rt-kKNG-for2	k-Kininogen HMW	CCAGAAGCAACTTCCCACA
rt-kKNG-rev2	k-Kininogen HMW	GCATCGAGGAGATCAAAATCA
rtLBP-for1	lipopolysaccharide binding protein	ATC GAG TCC AAG CTC CAG AA
rtLBP-rev1	lipopolysaccharide binding protein	GCT GTG ACT GGC AGA GTT TG
rtC1QB-for1	Complement Component C1Qbeta	AAG GAG AGA AAG GGC TCC CC
rtC1QB-rev1	Complement Component C1Qbeta	GAC CCT TGG GGC CAA CTT
rtCSPG4-for1	Chondroitin sulfate proteoglycan	TCC TGG AGA GAG GTG GAA GA
rtCSPG4-rev1	Chondroitin sulfate proteoglycan	CGA TCC ATC TCT GAG GCA TT
rtGPNMB-for1	glycoprotein (transmembrane) nmb	TTAATGCCTACTGGCCACAA
rtGPNMB-for1	glycoprotein (transmembrane) nmb	AATTGTGATGGTGGCTCTGA
rtIGFBP5-for1	Insulin-like growth factor binding protein 5	AAGCTTCCCTCCAGGAGTTC
rtIGFBP5-frev1	Insulin-like growth factor binding protein 5	AAGGCTTGCACTGCTTTCTC
rtLU-for1	Lutheran blood group (Auberger b antigen)	TGTTCCACTTTGGCTCTGTG
rtLU-rev1	Lutheran blood group (Auberger b antigen)	GCAGCGACTACGAGTAGCAA
rtNexelin-for1	Nexilin	AATGGGAGTAAGCCCCAAGT
rtNexelin-rev1	Nexilin	GGTTGGTTGGTTGGTTGTTT
rtPKD-for1	Polycystin 1 precursor	GCAGCACCTTCTTTTTGGTC
rtPKD-rev1	Polycystin 1 precursor	ACAAGGCAGCTTCATTTGCT
rtPLA1A-for1	Phosphatidylserine-specific phospholipase A1	GATTGCAGTGGACTGGGTTT
rtPLA1A-rev1	Phosphatidylserine-specific phospholipase A1	GCTCCAAAAGTTTGCTGAGG
rtRAD-for1	Ras-related associated with diabeties	ACATTTGGGAACAGGATGGA
rtRAD-rev1	Ras-related associated with diabeties	TTTCTCAAAGCTGCCCTTGT
rtTGFB3-for1	Tumor Growth Factor Beta 3	TTGCGGAGAGAGTCCAACTT
rtTGFB3-rev1	Tumor Growth Factor Beta 3	TTTCCATCACCTCGTGAACA
rtC3-for1	Complement component 3	CAAAACTGTGGCTGTCCGTA
rtC3-rev1	Complement component 3	TTGGTCACTGAGGTCTGCTG
rtICAM1-for2	ICAM-1 Intercellular adhesion molecule 1	CAGACCCTGGAGATGGAGAA
rtICAM1-rev2	ICAM-1 Intercellular adhesion molecule 1	GTGGGCTTCACACTTCACAG
rtMCP1-for1	MCP-1 Monocyte chemoattractive molecule 1	TAGCATCCACGTGCTGTCTC
rtMCP1-rev1	MCP-1 Monocyte chemoattractive molecule 1	CCGACTCATTGGGATCATCT
rtCARP-for1	Carbonic anhydrase related protein	AGTGGGGTTACGAGGAAGGT
rtCARP-rev1	Carbonic anhydrase related protein	AGGGGTCGTATCTGGCTTCT
rtNOS3-for1	endothelial nitric oxid synthase-3 (eNOS)	ATGGATGAGCCAACTCAAGG
rtNOS3-rev1	endothelial nitric oxid synthase-3 (eNOS)	GGATGCAAGGCAAGTTAGGA
rtMMP2-for1	Matrix-Metalloproteinase-2	ATGACATCAAGGGGATCCAG
rtMMP2-rev1	Matrix-Metalloproteinase-2	GGAGTGACAGGTCCCAGTGT
rtMMP9-for1	Matrix-Metalloproteinase-9	CGAGACCTGAAAACCTCCAA
rtMMP9-rev1	Matrix-Metalloproteinase-9	GCTTCTCTCCCATCATCTGG

Sequences of oligonucleotides used as forward or reverse primers for qRT-PCR.

2.9.2. Antibodies

Table 4. List of antibodies used for immunhistochemistry

Primary antibodies	**Company**	**Cat.No.**	**Dilution**
Mouse-PCNA monoclonal	Acris	SM 1421 P	1:50
Mouse anti-Ki 67 monoclonal	Dako	Mib 5	1:50
Mouse anti-Ki 67monoclonal	Dako	Tek 3	1:50
Mouse anti-CD 68 monoclonal	Acris	BM 4000	1:50
Mouse- anti-CD 68 monoclonal	serotek	MCA-341R	1:50
Cy3 mouse anti-SMC-Aktin monoclonal	Sigma-Aldrich	C-6198 (1A4)	1:100
rabbit anti-Kininogen heavy chain polyclonal	Santa Cruz	(H-70) sc-25799 (M-20)	1:50
rabbit anti-Kininogen light chain polyclonal	Santa Cruz	sc-25889	1:50
rabbit anti-Bradykinin antiserum	Bachem	T-4019	1:1000
Secondary antibodies	**Company**	**Cat.No.**	**Dilution**
Biotin goat anti-mouse IgG	DakoCytomation	E0432	1:100
Cy3 goat anti-mouse IgG	Amersham	PA43002	1:100
Cy3 goat anti-mouse IgG	Jackson Immuno Research	115-165-166	1:100
Cy3 goat anti-rabbit IgG	Jackson Immuno Research	111-165-144	1:100
Alexa 488 donkey anti-rabbit IgG	Molecular Probes	A-21202	1:100
Biotin goat anti-rabbit IgG	Vector Labs	BA-1000	1:100

3. Results

3.1. Characterization of cerebral arteriogenesis in the 3-VO model in the rat

In order to analyse molecular mechanism of early-phase cerebral arteriogenesis a nonlethal model of brain hypoperfusion was employed in rats (3-vessel occlusion model). For this purpose, the 3-VO model was established. Surgery did not lead to visible neurological defects. As well, no noticeable differences in behaviour were detectable on rats post surgery as compared to controls. 3-VO surgery is difficult to perform, and successful vertebral artery occlusion is not visible via the paraveterbal access. Therefore, successful occlusion had to be verified by Laser Doppler flow measurements (LDF). Blood flow was measured in the parietal cortex by LDF, before and following bilateral vertebral artery and unilateral carotide artery occlusion. 3-VO worked properly if blood flow values declined and remained at the 50 % level as compared to blood flow values before surgery, which is exemplified in Figure 9. As expected from adaptive brain arteriogenesis, blood flow recovers day to day until at day 21 it finally archives values comparable to the time-point before occlusion (data not shown).

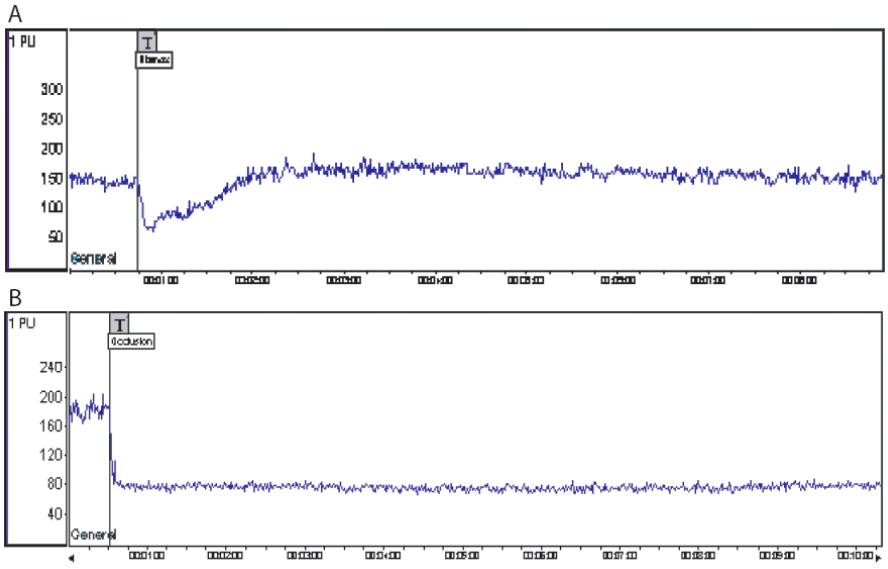

Figure 9. LDF measurement of the parietal cortex

Successful 3-VO surgery was verified by LDF. A: Prior to 3-VO, B: Following 3-VO blood flow drop to around 50 %. The 3-VO model represents a non ischemic hypoperfusion model.

After verifying a successful 3-VO procedure, it was confirmed that blood flow is redistributed via the circle of Willis leading to collateral artery growth in the brain. Validation of cerebral arteriogenesis was performed by the cerebrovascular latex perfusion method, which is also required to identify our area of interest for further

morphological and molecular biological studies. Cerebrovascular latex perfusion enables diameter measurements of vessels in a situation of complete vasodilation and subsequent vasoparalysis, and therefore reflects the true anatomic size of the vessels. An increase of the vessel diameter was analysed for three parts of the circle of Willis, including the anterior (ACA), the medial (MCA), and the posterior cerebral artery (PCA).

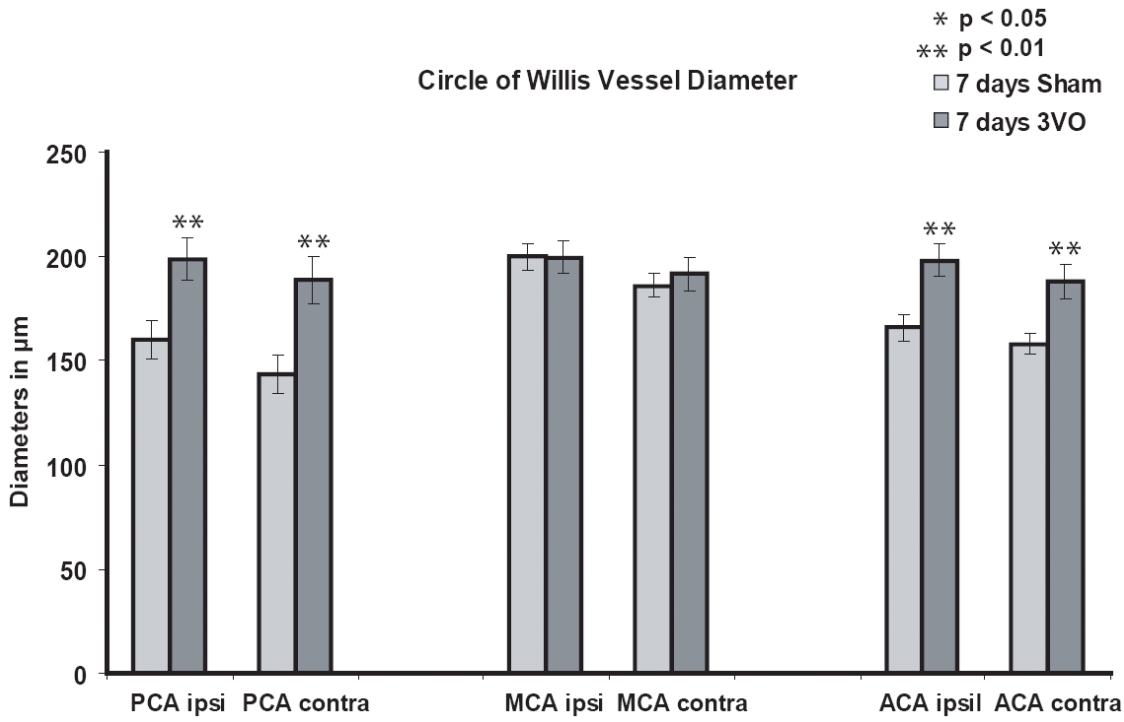

Figure 10. Vessel diameters in the circle of Willis 7 days post 3-VO or sham surgery

Vessel diameters following maximal vasodilation are given for the anastomoses (ACA, MCA and PCA) within the circle of Willis. Diameters are shown for the ipsilateral side and the contralateral side of the carotid artery occlusion.

Overall analysis of more than 25 brains per group indicate that application of a high dose of adenosin before latex perfusion produced maximal vasodilation that was unaffected by intravital differences in vascular tone. At 24h post 3-VO, no significant changes in the vessel diameter within the circle of Willis were detectable as compared to sham control animals (data not shown), indicating that this time-point is to early for collateral growth to become visibly detectable. However, as expected, increase of the vessel diameter was found in the circle of Willis, which reached significance at 7d post 3-VO as compared to sham operated controls (Figure 10). Here, the ipsilateral diameters post 3-VO increased significantly from 162 µm (+/- 40 µm) to 205 µm (+/- 45 µm).

Contralaterally, diameters increased from 147 µm to 196 µm in comparison to sham-operated controls. No significant morphological differences were detectable between ipsi- and contralateral regions of the PCA. At 7d post 3-VO diameter of the ACA ipsilateral showed changes from 166 µm to 198 µm; the contralateral ACA showed diameter increases from 158 µm to 188 µm as compared to sham control animals. Likewise to the PCA region no significant morphological differences are detectable between ipsilateral and contralateral regions of the ACA. However, a trend indicating that the ipsilateral region grew as compared to the contralateral region is detectable, although the differences are not significant. Finally, vessel diameter of the MCA region do not show differences between 3-VO animals and sham control animals.
In summary, the PCA show strongest collateral growth in the circle of Willis and it is hypothesized that the PCA is the region of interest for analysing arteriogenesis.

3.1.1. Histological evaluation of morphological features of arteriogenesis

Given that the PCA is recruited as a collateral pathway upon 3-VO to supply the hypoperfusion area with blood, enhanced blood flow would induce arteriogenesis and morphological features of collateral remodelling should be detectable. Morphological features of arteriogenesis were analysed by immunhistological methods to further characterize major parameters of arteriogenesis, such as active cell proliferation and macrophage invasion. In subsequent experiments, all antibodies were tested on paraffin sections from brain with longitudinal sections from the PCA region.

First of all, different commercially available antibodies directed against proliferation markers such as Ki67 and PCNA were used. Since PCNA has a longer half-life in mitoses than Ki67, it was decided to use an antibody for PCNA to screen for cell proliferation in the PCA. Using a PCNA antibody, an increase in vascular cell proliferation 24h and 3d after 3-VO was detectable (Figure 11A-D), demonstrating that cell proliferation already occurs in early-phase cerebral arteriogenesis.

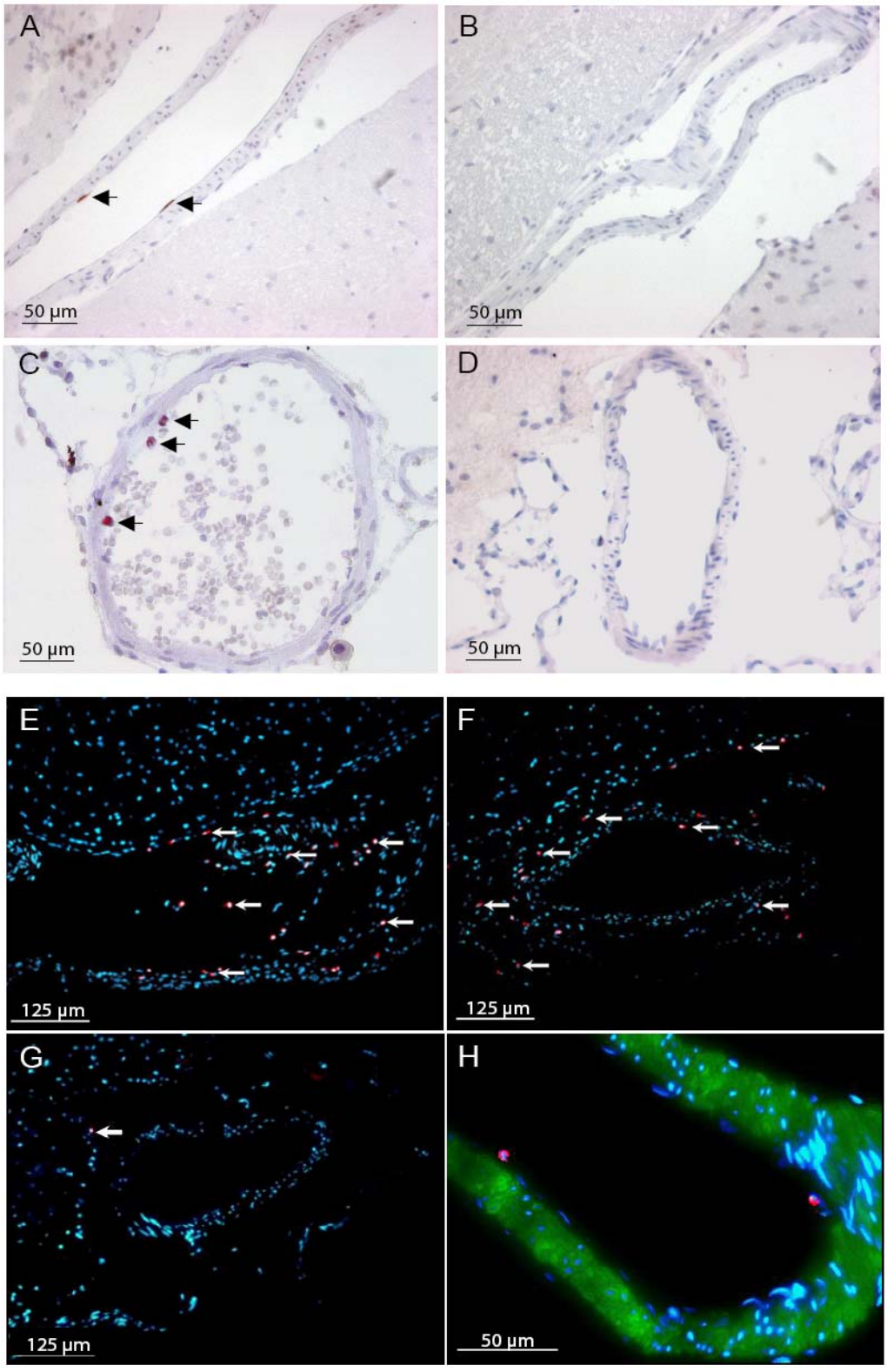

Figure 11. Immunostaining for PCNA and CD68 in the PCA region

A-D: Staining for proliferation marker PCNA (red). Hematoxylin staining of nucleus (blue) A: PCA region 24h post 3-VO surgery, B: PCA region 24h post sham surgery, C: PCA region 3 days post 3-VO surgery, D: PCA region 3 days post sham surgery. E-H Staing for macrophage marker CD68 (red). Hoechst staining of the nucleus (blue). Staining for smooth muscle actin (green). E: PCA region 24h post 3-VO surgery, F: PCA region 3 days post 3-VO surgery, G: PCA region 24h post sham surgery, H: Magnification of PCA region 24h post 3-VO surgery.

Only a scarce distribution of proliferating cells was detectable on sections from sham control animals. Differences in numbers of proliferating cells between the posterior cerebral arteries on the ipsi- or contralateral side of the ligated carotid artery could not be observed, showing that cell proliferation in the PCA occurs bilaterally, which is in line with the findings for PCA diameter growth. Interestingly, a closer look on the histological sections reveal that 3 days after 3-VO endothelial cells seem to bulge into the lumen (Figure 11, Arrow), which is neither detectable 24 hours after 3-VO nor on sections of sham control animals.

Second, antibodies were applied against the macrophage marker CD68 (ED-1 in rat) to analyse for enhanced monocyte transmigration in the PCA in early-phase cerebral arteriogenesis. As described in section 1.3., macrophage invasion is an important process for inducing arteriogenesis. Indeed, CD68 staining showed a significantly increased number of invading macrophages 24h and 3d post 3-VO (Figure 11E, 11F) in the perivascular space compared to controls (Figure 11G). Accumulation of CD68-positive cells was detected in the adventitia as well as in the surrounding arachnoidea in close proximity to the growing PCA. Positive CD68 staining was also obtained at the endothelial cell layer, indicating adhesion of macrophages to endothelial cells (Figure 11H).

In summary, histological analysis of the PCA (24h and 3 days) post 3-VO reveal typical features of arteriogenesis.

3.1.2. Electronmicroscopical analysis of flow activated endothelial cells

Enhanced blood flow via the PCA post 3-VO, results in increased fluid shear stress. Fluid shear stress is the driving force of arteriogenesis (section 1.3.), resulting in endothelial cell activation. Activation of endothelial cells by enhanced blood flow in the growing collaterals of early-phase arteriogenesis was analysed by applying scanning electron microscopy. Thus, 24 hours and 3 days after 3-VO or sham surgery PCA vessels were selectively removed, and a novel technique was developed to prepare the PCA for microscopy (see section 2.4. and 4.1.4.). The PCA was opened lengthwise using iris scissors, and different tissue glues were tested to adjust the opened vessel on a glass slide (Figure 12A, 12B). Second, critical point drying in carbon dioxide and subsequent spotting with ionized gold particle under an argon atmosphere allowed visualization of the endothelial cell layer.

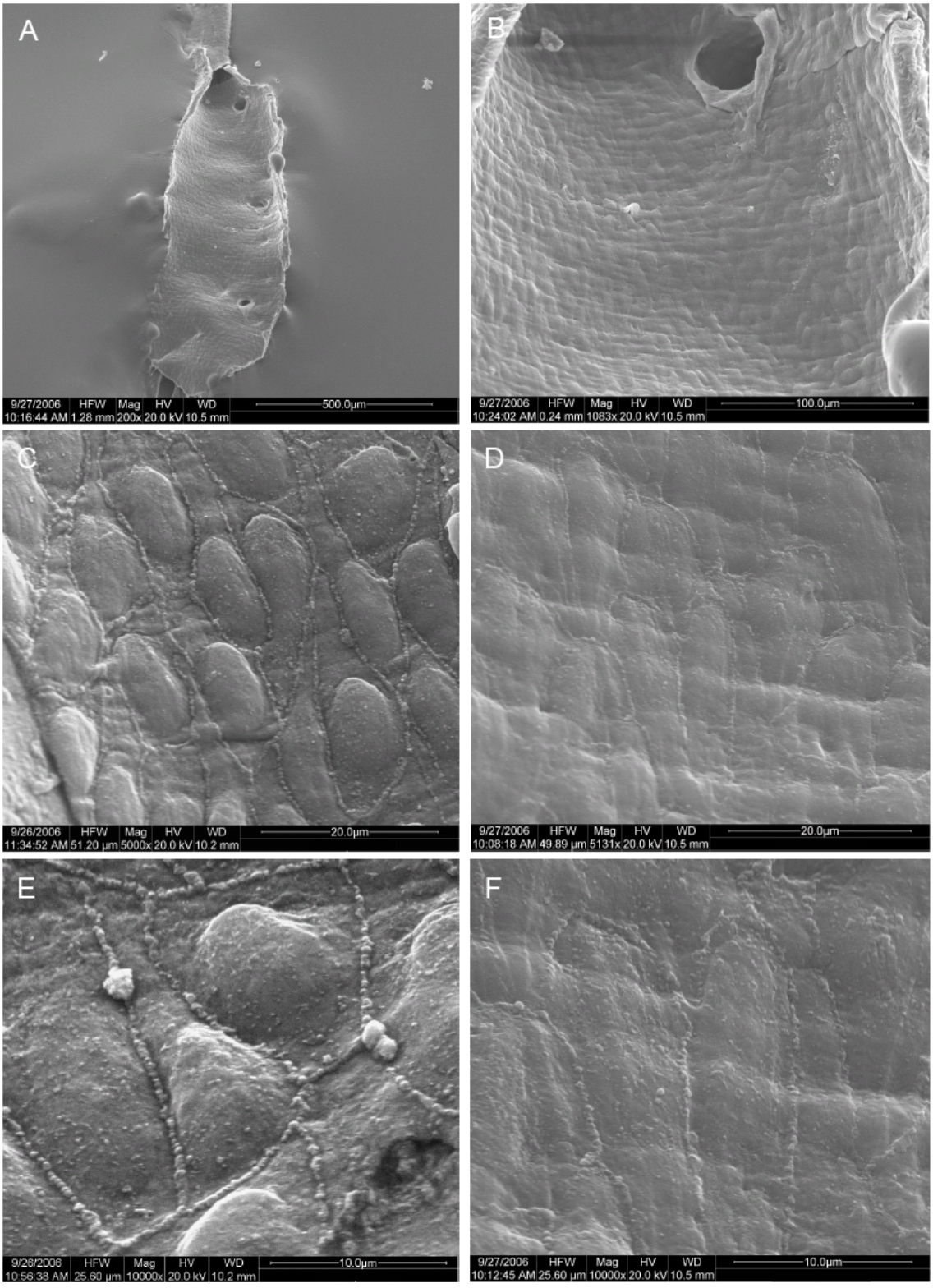

Figure 12. Scanning electron microscopy of the endothelial layer of the PCA

A: PCA is opened lengthwise and mounted on glass slide. B: Microscope zoom allows visualization of the endothelial cell layer. C: 3 days post 3-VO surgery (5000x), D: 3 days post sham surgery (5000x), E: 3 days post 3-VO surgery (10.000x), H: 3 days post sham surgery (10.000x).

Scanning electron microscopy, visualizes the PCA endothelial cell layer of sham animals, which exhibit a smooth and flat cellular surface (Figure 12D, 12F, previous page). In comparison, a cobblestone-like pattern was detectable in the growing PCA 3d

post 3-VO (Figure 12C, 12E). On closer inspection, the endothelial cells reveal that this cobblestone-like pattern is formed by a protrusion of the nucleus. In addition, endothelial cells of the PCA 3d post 3-VO are divided by a demarcation line. Here, cell borders show pronounced structures, and is in contrast to sham animals. Furthermore, at 3d post 3-VO, big globular-like objects are frequently visible between cellular borders. At 24h post surgery, the endothelial cell layer of the PCA in 3-VO animals was smooth and flat and comparable to the endothelial cell layer of sham animals (data not shown).

3.2. Molecular mechanisms of early-phase cerebral arteriogenesis

Vessel diameter measurements and morphological characterization of growing collaterals identified the PCA in the circle of Willis as the region of interest, which showed typical features of arteriogenesis; however, little is known about the molecular mechanisms of early-phase cerebral arteriogenesis. Thus, the PCA was chosen for further analysis of the molecular mechanism of early-phase cerebral arteriogenesis. Despite challenging anatomical conditions in the brain, the 3-VO model permits the selective preparation of a growing collateral artery (PCA), which is located on the surface of the brain (section 1.5). This study describes the first genomic approach focused on a selective isolation of a growing collateral artery and managed to harvest the PCA 24 h and 3 days after 3-VO. Subsequently, RNA was isolated for identification of differentially expressed genes during adaptive cerebral arteriogenesis (Figure 13).

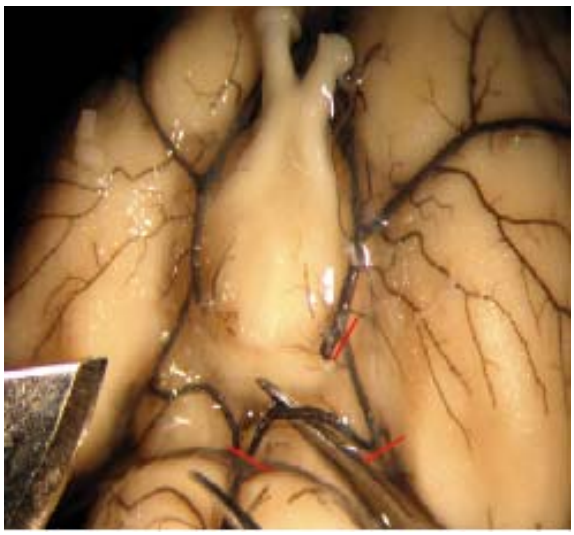

Figure 13. Selective isolation of the PCA out of the circle of Willis in the rat brain

Angiography of the circle of Willis in the rat brain. Vessels of the area of interest (PCA/Pcom) which were isolated are marked by red bars. Vessels were swiftly isolated and immediately stored in `RNA later´ (Ambion) for RNA preservation.

3.2.1. Genomic profiling

A global genomic profiling approach was performed using the Affymetrix chip technology (section 2.4.4) to analyse the patterns of the initial start up phase of arteriogenesis. At NCBI Gene Expression Omnibus, raw data of the chip hybridisation experiment are available, where series record GSE6189 provides access to all expression data. At 24h post 3-VO, significance analysis of microarrays (SAM) identified 91 genes as upregulated and 73 genes as downregulated (n=164 out of 15.866 probe sets) as compared to sham-operated controls. Expression differences are considered significant, as SAM integrates for each gene random permutations of the transcription values of all samples and the expression deviation between biological replicas.

However, global gene expression profiles raise the problem that extreme amounts of data are produced without any basic concept. In order to understand molecular patterns of early-phase arteriogenesis and to identify relevant target genes, which have functional relevance for arteriogenesis, several bioinformatics software tools were used to structure the various genomic data.

First, genes identified by SAM as deregulated were grouped into genetic networks by knowledge-based analysis of physical, transcriptional, or enzymatic interactions by the Ingenuity Pathway Analysis software tool (IPA). Here, IPA demonstrates that gene expression of cerebral arteriogenesis 24h post 3-VO can be clustered into six functionally related networks. Networks designed by IPA are presented in Figure 14 (next page) showing deregulated genes, where colors indicate the degree of increased expression in red, decreased expression in green, and unregulated gene expression in white. Network 1 comprises genes involved in cellular movement and cellular development. Genes in network 2, 3, and 5 are related to cellular growth, cellular proliferation, and cancer. The functional clustering to cancer development might be confusing. However, this relation make sense, as genes shown here are responsible for angiogenesis and vessel formation as a basic mechanism for cancer development, and, interestingly, reappear in collateral development. Furthermore, Network 3 is relevant for cardiovascular system development. Networks 4 and 6 show genes related to inflammatory processes and molecular transports.

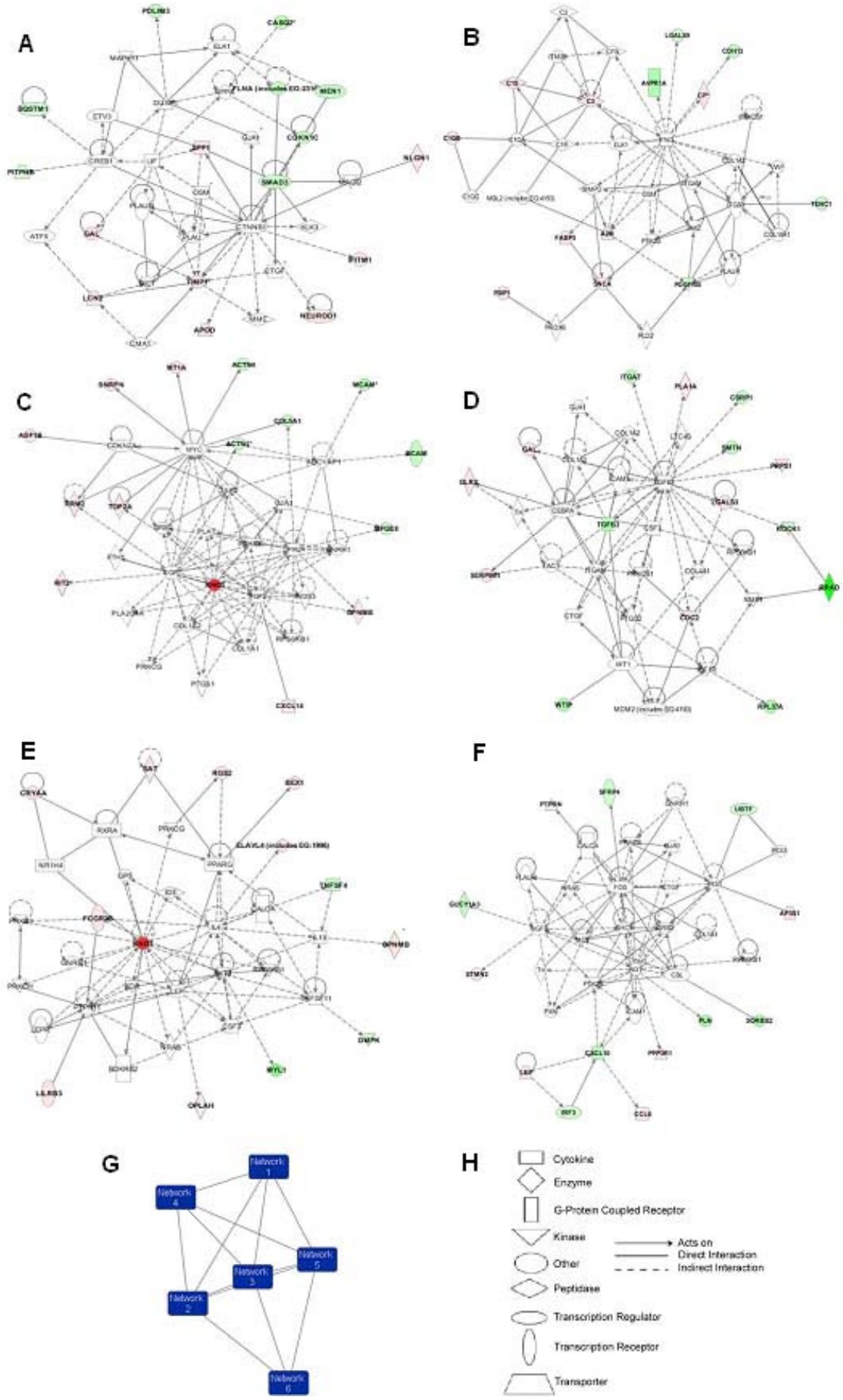

Figure 14. Ingenuity network analysis of early-phase cerebral arteriogenesis

Identification of transcriptional, enzymatic or physical interaction of deregulated genes 24h post 3-VO.

Network analysis shows that all six functional networks are related to each other on a superordinate level and to cardiovascular system development and function (Figure 14H). Second, molecular analysis for patterns of early-phase of cerebral arteriogenesis aimed at summarizing expression values of those genes involved in the six distinct networks. Therefore, all genes were clustered into groups corresponding to the networks and expression values visualized by GeneMath for each biological replicate, as presented in Figure 15 (next page) together with the according fold change value (24h post 3-VO).

Visualized values of those genes demonstrate homogeneous distribution within the groups (Figure 15), indicating the high reliability of the expression data. Although 3d post 3-VO, most genes were not significantly deregulated; graphical visualization of listed genes shows clearly a similar trend of genes 24h post 3-VO and 3d post 3-VO. At 24 hours post 3-VO, differently expressed genes show that highest expression values were between 3 and 4 fold change deregulated. The following genes are to be mentioned in this context: *secreted phosphoprotein* (network 1), *phospholipase A1 member A* (network 2), *serin phospholipase A1* (network 2), *glycoprotein nmb* (network 3, 6), *methallothionein* (network 3), *topoisomerase II alpha* (network 3), *chemokine ligand 14* (network 3), *ceruloplasmin* (network 4), *alpha-2 macroglobulin* (network 4), *complement component 3* (network 4), *chemokin-ligand 6* (network 5), *crystallin alpha A* (network 6), *FC fragment of IgG* (network 6) and *leucocyte Ig-like receptor B member 3* (network 6) (Figure 14, Figure 15). For more detailed analysis and literature research affymetrix identifiers for all genes are given in Figure 15 as well.

Moreover, in particularly noticeable at 24h post 3-VO is the expression of *kininogen*, which is the most strongly upregulated gene in this expression profile (66.4 fold change) and is involved in networks 3 (cellular growth and proliferation) and 6 (inflammation) (Figure 15). *Lipocalin 2* and *Tissue inhibitor of metalloproteinase-1* (network 1) showed second and third highest expressions, respectively, and are upregulated more than 6 fold.

In summary, IPA analysis demonstrates that genes deregulated in early-phase cerebral arteriogenesis are part of related networks assigned to three major functions or categories: cellular movement, inflammationy and cellular growth/proliferation (Figure 15).

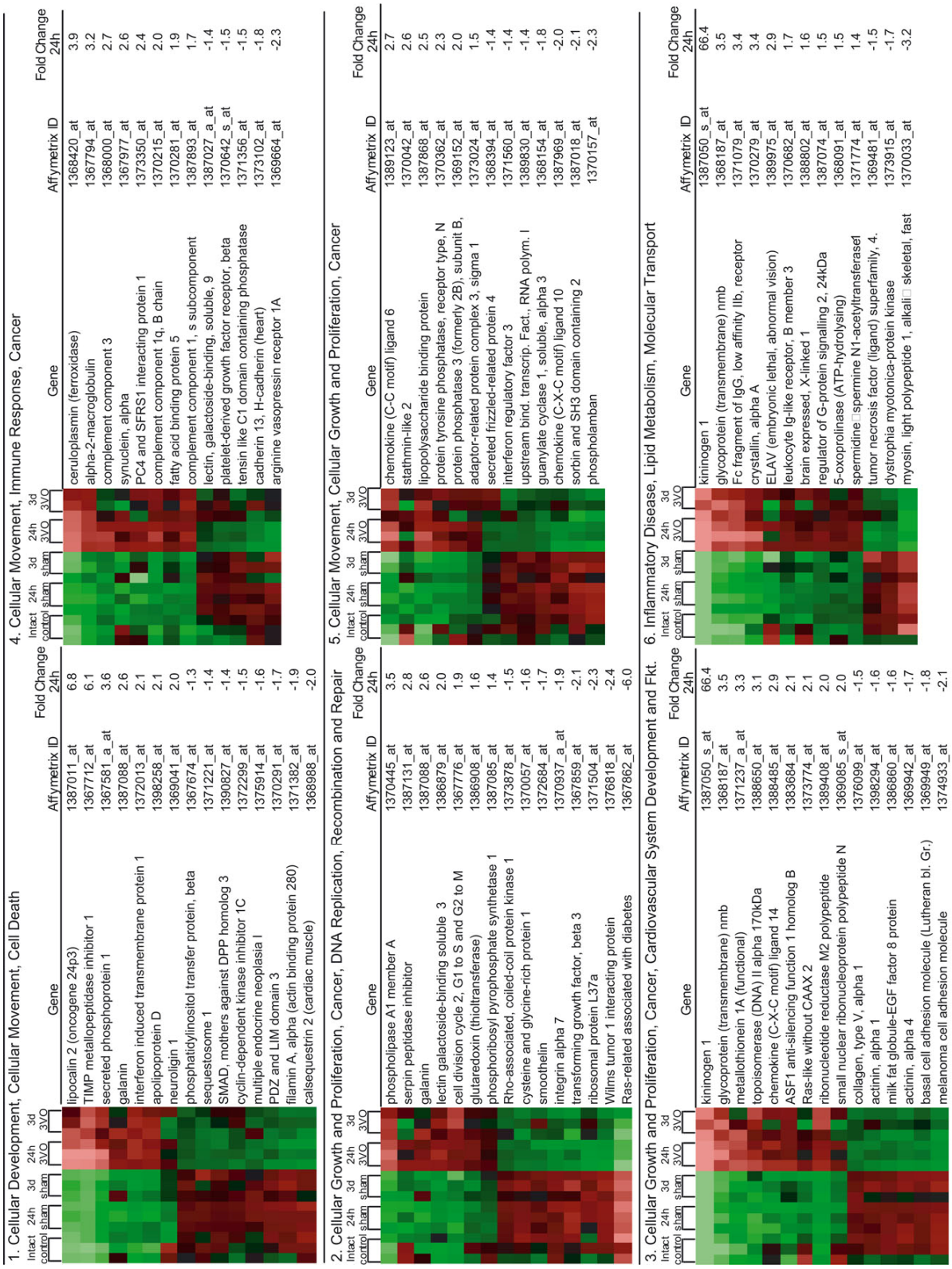

Figure 15. Genomic profile of early-phase cerebral arteriogenesis

Visualization of the genomic expression profile. Genes deregulated in early-phase cerebral arteriogenesis are clustered in 6 functional networks related to cardiovascular system development. Figure shows upregulated genes in red and downregulated genes in green. Expression profile shows homogeneous distripution within the groups. Fold change values are given 24h post 3-VO surgery as compared to sham controls. Affymetrix identifiers are given for gene specification.

The overall expression profile so far indicates a common pattern for vascular remodelling, obtained from angiogenesis and for arteriogenesis, which points to a number of highly upregulated regulatory genes involved. Indeed, those patterns provide the first clue about the mechanisms of early-phase cerebral arteriogenesis.

To further analyse those genes involved, a detection software for overexpressed Gene Ontology (GO) -categories was used (DAVID database). The DAVID Bioinformatic database was applied to annotate all known genes involved in the network, as shown above (Figure 15) to biological function and molecular function.
Biological function annotation summary showed that highest significance levels were assigned to the GO-categories `response to pathogens´, `response to wounding´, `immune response´, and `defense response´ (Table 5A). In conclusion, early-phase cerebral arteriogenesis includes many genes involved in inflammatory processes. Detailed list of all genes annotated to the most characteristic GO-categories are given in the appendix (Page 94). Studying GO-categories more closely showed, for example, genes involved in inflammatory processes are: *alpha-2 macroglobulin*, *chemokine ligand 14*, *complement components 1* and *3*, *lipoprotein binding protein*. In this context, it is notable that some genes are grouped to the GO-categories `Mononuclear cell proliferation´ and `leukocyte mediated immunity´. Mononuclear leucocyte recruitment and proliferation is a major feature of arteriogenesis, and here genes are involved, such as *kininogen* and *complement component 1, q subcomponent, beta polypeptide.*
Frequently, genes were annotated to multiple functions, as was *kininogen*, which is likewise functionally annotated to `cell differentiation processes´ and *Tissue inhibitor of metalloproteinase-1 (TIMP-1),* which is likewise functionally annotated to `regulation of cell proliferation` and `system development´. Biological function annotation also show that 24h post 3-VO many genes are related to processes, such as `cell proliferation´, `cell differentiation´ and `system development´. To quote an example these functional groups contained genes, such as *interferon induced transmebrane protein 1*, *glycoprotein nmb* and surprisingly neuronally related genes, such as *neurogenic differentiation 1*, and *semaphorin 4f.*

Biological function annotation to significantly repressed genes showed several genes related to `muscle development´ and `muscle contraction´ (Table 5B), such as *smoothelin* and *myosin light chain*. Similar to biological processes identified from

upregulated genes, some downregulated genes (basically inhibitors) were functionally related to processes, such as `system development´ and `cell proliferation´ as well.

Table 5. DAVID biological function annotation summary

A Biological function annotation

Genes positiv regulated 24h post 3-VO	No. of genes	p-value
response to wounding	12	1,70E-05
inflammatory response	10	2,30E-05
system development	20	9,30E-05
adaptive immune response	5	1,00E-03
response to hormone stimulus	6	4,10E-03
regulation of immune response	5	6,80E-03
regulation of immune system process	5	8,10E-03
negative regulation of biological process	12	1,40E-02
mononuclear cell proliferation	4	1,90E-02
cell differentiation	15	2,20E-02
leukocyte mediated immunity	4	2,30E-02
regulation of cell activation	4	2,40E-02
regulation of multicellular organismal process	7	2,70E-03
negative regulation of cellular process	11	2,90E-02
regulation of cell proliferation	8	3,30E-02
neurogenesis	7	3,50E-02
humoral immune response	4	3,70E-02
positive regulation of biological process	11	6,40E-02
organ development	12	7,90E-02

B Biological function annotation

Genes negative regulated 24h post 3-VO	No. of genes	p-value
muscle development	6	1,10E-03
muscle system process	5	1,50E-03
circulatory system process	5	3,40E-03
embryonic development	6	3,80E-03
regulation of cell proliferation	7	7,00E-03
organ development	11	8,40E-03
system development	13	9,60E-03
positive regulation of cellular process	9	1,40E-02
positive regulation of biological process	9	2,40E-02
regulation of cell motility	3	2,60E-02
regulation of locomotion	3	3,00E-02

A: Biological function annotation of genes upregulated. B: Biological function annotation of genes downregulated. Genes upregulated annotated highly significant to biological functions related to inflammatory processes. Upon genes downregulated most were significant related to muscle development and construction.

In summary, biological function annotation using DAVID bioinformatic database demonstrates that the main focus of early-phase cerebral arteriogenesis is on

inflammatory processes. Furthermore it is shown, that 24h post 3-VO cellular proliferation, cellular differentiation and de-differentiation are relevant processes for collateral growth.

Table 6. DAVID molecular function annotation summary

C Molecular function annotation	No. of genes	p-value
Protease inhibitors	4	7,10E-04
Immunoglobin binding	2	5,10E-03
Receptor binding	6	6,70E-03

Protease inhibitor activity
Alpha-2-Macroglobulin
Kininogen
Tissue inhibitor of metalloproteinase 1
Serine (or cysteine) peptidase inhibitor, I1

Annotation for molecular function of deregulated genes revealed a very significant cluster for protease inhibitors.

Finally, annotation of deregulated genes to molecular function at 24h post 3-VO revealed a significant cluster of protease inhibitor genes of which kininogen, alpha-2 macroglobulin (A2M) and Tissue inhibitor of metalloproteinase-1 (TIMP-1) had lowest p-values (0,00071, Table 6). Genes of these protease inhibitors show the strongest deregulation and are involved in most functional networks generated by Ingenuity (Figure 14, 15). *In silico* analysis and DAVID database analysis clearly show that protease inhibitors are multifunctional proteins, involved in most processes of cardiovascular system development (see above), and are expressed in many physiological and pathological conditions. Hence, protease inhibitors are investigated in more detail in the following paragraph.

Protease inhibitor Kininogen (66.1 fold change, Figure 15), by far the strongest gene expressed in early-phase cerebral arteriogenesis regulates the homeostasis of extracellular matrix digestion in vascular remodelling. Furthermore kininogen is the substrate of kallikrein which liberates the highly vasoactive compound bradykinin (section 1.8.).

Likewise, TIMP-1 (6.1 fold change, Figure 15), the third most upregulated gene in the expression profile, is a protease inhibitor responsible for regulating matrix metalloproteinase activity. Lipocalin 2 (LCN2), a non-protease inhibitor and the second most deregulated gene in this profile (6.8 fold change, Figure 15), is shown to be

functionally related to TIMP-1 in extracellular matrix degradation. TIMP-1 also regulates the digestion of extracellular matrix in remodelling and exhibits growth factor activity, involved in other processes like modulating cell proliferation, migration, and leucocyte survival.
Finally, in network 3, expression of alpha-2 macroglobulin (A2M), another protease inhibitor, is reported, which is also involved in inflammatory processes, and was shown to be upregulated 3.2 fold 24h post 3-VO (Figure 15).

In conclusion, for those protease inhibitors, a multitude of functions are indicated and therefore protease inhibitors and related proteins were further validated by molecular analysis.

3.2.2. Promoter analysis

Data obtained in this study help to understand the molecular mechanisms of early-phase cerebral arteriogenesis and to identify genes, which may be involved in initiating collateral growth, but one important step is still not well understood. Mechanical force exerted by blood flow activates gene expression in endothelial cells, however, the following main driving stimulus governing the expression of relevant key players and maker genes of early-phase cerebral arteriogenesis is not yet known. Do forces acting on the endothelium induce expression of genes in a paracrine manner leading to inflammatory processes, or is gene expression 24h post 3-VO continuously directed by a flow-induced process?

Hence, a promoter analysis was performed for 53 most strongly deregulated genes, to identify inflammation-regulated genes by their NF-kB binding sites or flow-regulated genes by their shear stress response elements (SSREs). Therefore, a region from 5000 bp before to 1000 bp after the transcription start site of target genes was browsed for putative NF-kB binding site (inflammatory regulation) and SSREs (flow-mediated regulation).

Of 53 deregulated genes, promoter analysis identified 42 genes that contained at least one NF-kB binding site and only 5 genes containing at least one SSRE, which are listed in Table 7. However, all genes containing SSREs as well exhibit one NF-kB binding site. Noticeable is the *TGF-beta 3* gene, which was 0.48 fold downregulated exhibits 3

SSREs and 2 NF-kB binding sites. Furthermore, a gene related to neurogenesis, such as *Neurologenic differentiation factor 1* exhibit a SSRE.

Among our strongly deregulated proteinase inhibitors *Kininogen*, *TIMP-1* and *A2M*, and the functionally related *Lipocalin 2*, all exhibited more than one NF-kB binding site and non of them an SSRE.

Table 7. Promoter analysis

Sam Analysis 24h 3VO	positive deregulated	negative deregulated	total
10 % False Positive Rate	**73**	**91**	**164**
Promoter Analysis of 53 Potential Candidate Genes	positive deregulated	negative deregulated	total
Genes with NF-kB binding site	**27**	**15**	**42**
Shear Stress Response Elements	**3**	**2**	**5**
Promoter Analysis of Target Genes	fold change (Array analysis)	Number of NF-kB binding sites	Number of SSRE
Kininogen	**66.37**	**3**	**/**
TIMP-1	**6.06**	**9**	**/**
Lipocalin 2	**6.76**	**2**	**/**
Alpha-2-Macroglobulin	**3.21**	**4**	**/**
Neurogenic differentiation factor 1	**2.59**	**2**	**1**
ELAV-like protein 4/ Hu-antigen D	**2.91**	**2**	**1**
l-gicerin	**2.69**	**3**	**1**
similar to Small inducible cytokine A6 (CCL6)	**0.52**	**1**	**1**
Transforming growth factor beta 3 (TGF-beta 3)	**0.48**	**2**	**3**

Promoter analysis was performed for 53 early-phase cerebral arteriogenesis target genes and promoter region browsed for NF-KB binding sites or SSREs. Most candidate genes have at least one NF-kB binding site within their promoter region. Only 5 candidate genes exhibit a putative SSRE in the promoter region. However, all genes exhibiting an SSRE have as well at least one NF-kB binding sites in the promoter region.

Due to the overall impact of inflammation in early-phase cerebral arteriogenesis and the fact that most genes contained at least one NF-kB binding site, it was decided to continue molecular analysis by focusing on protease inhibitors. Here, strong expression values and the potential use for a therapeutical stimulation of arteriogenesis have been considered as well for the decision to study protease inhibitors in more detail.

3.2.3. Inflammatory Processes

Many genes involved in early-phase arteriogenesis were clustered and annotated to inflammatory processes, which is in line with the expression profile of Lee et al. for the initial phase of arteriogenesis in the mice hind limb. However, it was considered that

inflammatory genes are deregulated because of a systemic post-operative acute phase reaction. Each form of injury or tissue damage inevitably also causes an inflammatory response which may result in an acute phase reaction. An acute phase reaction is characterized, among other things, by an increase in the numbers of circulating white blood cells (e.g. neutrophils and macrophages). Furthermore, the liver responds by producing a large number of acute-phase reactants, such as C-reactive protein and serum amyloid A (SAA). To rule out acute phase reaction as a relevant cause of changes in the gene expression profiles, white blood cell count (WBC) was performed and rat serum amyloid-alpha (SAA) was determined, which correlates with the human CRP.

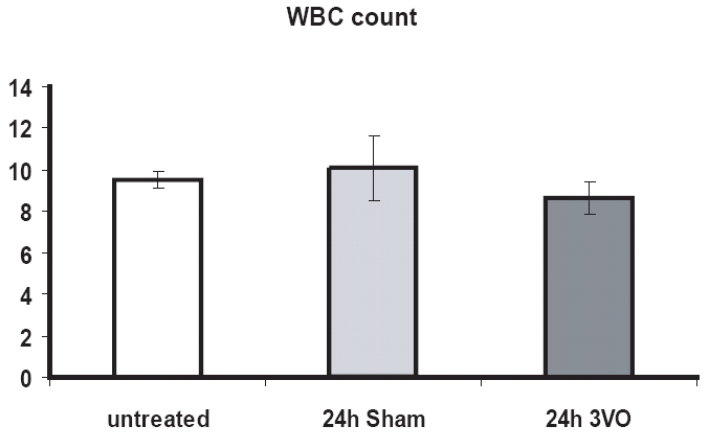

Figure 16. White blood cell count
Figure indicates that white blood cell number are unchanged whithin all animals comprised in the study for early-phase cerebral arteriogenesis (24h post 3-VO).

WBC was not altered in 3-VO animals and control animals after 24h and 3d post 3-VO (Figure 16). Serum amyloid alpha was below detection limit in all groups (data not shown).

3.2.4. Validation of target genes significantly deregulated in the PCA at 24h post 3-VO

Quantitative real-time RT-PCR was carried out with the same RNA samples used for microarray analysis to validate the results of the microarray analysis. Figure 17 shows expression levels of the protease inhibitors *kininogen*, *TIMP-1, A2M,* and the non-protease inhibitor *LCN2* as obtained by qRT-PCR, which validated the array hybridisation data and confirmed a significant increase in mRNA levels during early cerebral arteriogenesis in the ipsilateral PCA 24h post 3-VO compared to the sham group (Figure 17A-D).

More than 10 genes, which were found deregulated by SAM analysis, were validated by qRT-PCR. All confirmed the expression level shown by the affymetrix array hybridisation experiment (data not shown), indicating the high reliability of data obtained from the chip hybridisation experiment. In addition, gene expression levels for *kininogen*, *TIMP-1*, *A2M,* and *LCN2* were analysed by qRT-PCR in tissue samples from the PCA contralateral to the ligated carotid artery. Here, the increase in mRNA expression levels of all four genes was found to be similar to the one in the ipsilateral PCA region.

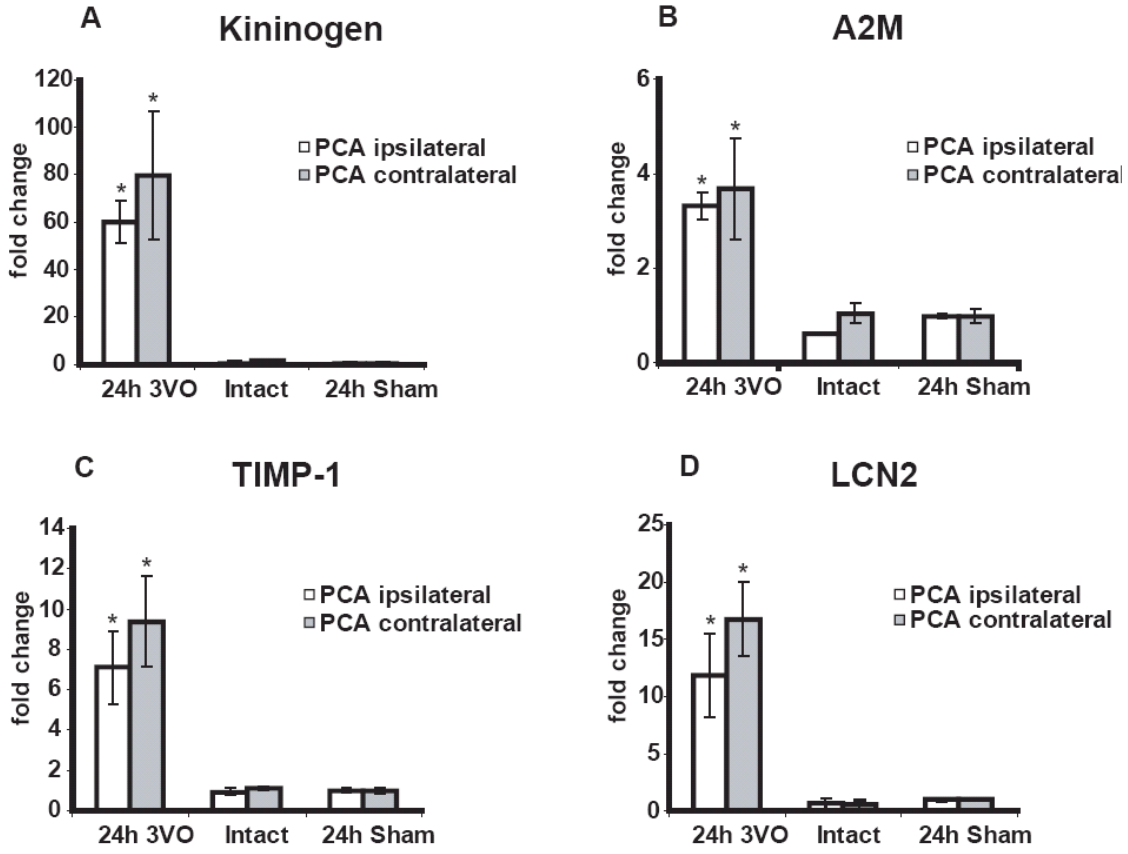

Figure 17. Validation of target gene expression by qRT-PCR

Validation of mRNA expression of the protease inhibitors kininogen, TIMP-1 and A2M and the related protein lipocalin 2. qRT-PCR was performed for RNA obtained from the ipsilateral and contralateral site of the carotid occlusion.

In order to control reliability of the genomic profile, expression of sham-operated animals and intact controls were compared. No significant expression differences were detectable (Figure 17). Importantly, expression levels of genes validated by qRT-PCR within each of the three replicates of each experimental condition were upregulated in a similar range, indicating a similar expression pattern within the same groups.

As protease inhibitor expression was validated by qRT-PCR, this study aimed at localizing potential target gene expression in early-phase cerebral arteriogenesis 24h post 3-VO. Probes for kininogen, A2M, TIMP-1, and Lipocalin 2 mRNA were generated for *in situ* hybridisation, which allows localization of mRNA expression in the vascular tissue of the PCA region. Here, it was managed to localize mRNA expression for *TIMP-1*, which recently was identified as being upregulated during arteriogenesis in the dog heart (Pipp, et al., 2004) and postulated to be a marker of coronary arteriogenesis. Furthermore, kininogen mRNA expression was localized by *in situ* hybridisation, since kininogen was one of the most strongly induced genes we identified. For *Lipocalin 2* and *A2M* it was not managed to show mRNA expression in the growing PCA.

In situ hybridisation showed a high TIMP-1 and kininogen mRNA staining intensity in the vascular wall of the PCA 24h post 3-VO (Figure 18A, 18D) compared to sham control (Figure 18B, 18E).

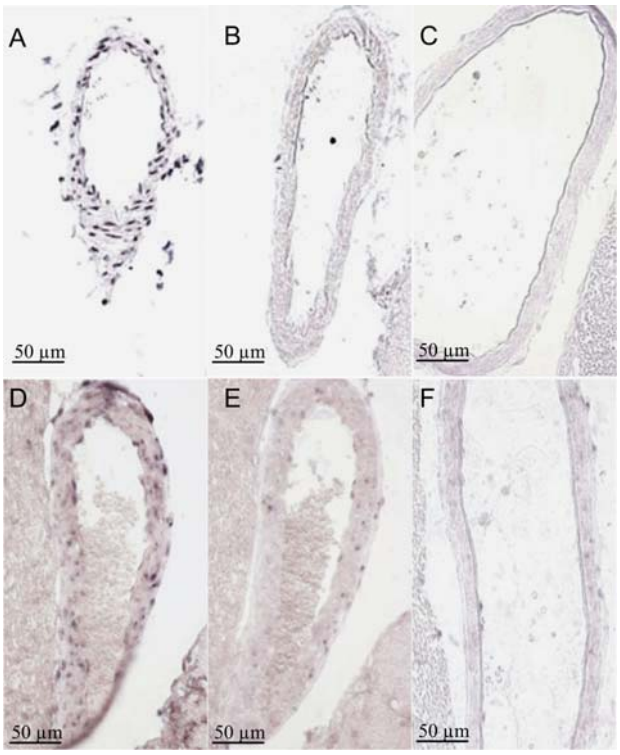

Figure 18. Localisation of target gene expression in the growing PCA post 3-VO surgery by *in situ* hybridisation

In situ hybridisation using DIG labled probes for TIMP-1 and kininogen mRNA on rat brain paraffin sections showed a positive signal in the vascular wall of the PCA 24h post 3-VO.
The expression of TIMP-1 and kininogen is detectable in endothelial cells, smooth muscle cells and tissue of the arachnoidea.

Increased expression levels of TIMP-1 and kininogen mRNA were detected in endothelial cells, smooth muscle cells, and adjacent meningeal tissues (arachnoidea).

Using sense probes for TIMP-1 and kininogen, no staining was detectable (Figure 18C+18F), indicating specificity of the probes.

In summary, molecular analysis of growing collaterals (PCA) in the brain identified kininogen and TIMP-1 as strong deregulated gene within the vessel wall 24h post occlusion. Excessive data base research, *in silico* analysis, and the use of bioinformatic databases implicate a multifunctional role of kininogen and TIMP-1, which are involved in many biological pathways. Promoter analysis indicates regulation of inflammatory process in the context of kininogen and TIMP-1 gene expression, and previous studies already demonstrate that arteriogenesis is initiated by inflammatory processes (Buschmann, et al., 2003). In this study, protease inhibitor mRNA expression was validated by qRT-PCR and kininogen, and TIMP-1 mRNA expression was localized in the growing PCA 24h post 3-VO, which identified Kininogen and TIMP-1 as marker of early-phase cerebral arteriogenesis.

3.3. Functional Analysis of the relevance of the kininogen signaling pathway for arteriogenesis

Kininogen and TIMP-1 were identified as marker genes of early-phase cerebral arteriogenesis. The third part of this work is intended to give insight into the functional relevance of a newly identified target gene for collateral growth. For many reasons, kininogen appeared to be a much more attractive target and was chosen as target for functional analysis. In particular, kininogen-bradykinin signaling is involved in vasodilation, in inflammatory processes, in leucocyte recruitment, and in processes of angiogenesis (section 1.8.). However, the bradykinin signaling pathway has not been analysed before in the context of arteriogenesis. Since this is a study based on a genomic analysis, it is much more reliable, analysing signaling pathways on transgenic lose of function models, than using pharmacological inhibitors. Hence, studies were performed by using the bradykinin receptor B1, the bradykinin receptor B2, and a newly developed strain with a double KO of the bradykinin receptor B1 and B2. However, the problem arose that the 3-VO model is yet not applicable to mice, for technical reasons (section 1.5). To ascertain the involvement of the kininogen-bradykinin signaling in arteriogenesis, the technically available femoral artery occlusion model was used. The femoral artery occlusion model is the best-characterized model for analysing arteriogenesis and is regularely used for the purpose of *in vivo* verification of data obtained in arteriogenesis research. In particular, the femoral artery ligation model

enable the direct measurement of arteriogenesis by the microsphere technique and perfusion (van Royen, et al., 2002) (section 1.6.).

3.3.1. Kininogen expression is enhanced in hind limb arteriogenesis

Certainly, one could expect differences in the molecular mechanism of arteriogenesis in the brain and the hind limb, however, this study is merely intended to answer the question whether kininogen has a fundamental relevance for processes of collateral growth in general. To identify whether kininogen mRNA expression in early-phase arteriogenesis is comparable to early-phase arteriogenesis in the brain, qRT-PCR was performed on RNA isolated from complete adductor muscle of the ligated and unligated hind limb. The adductor muscle contained the collaterals in the hind limb, which make selective isolation of collateral vascular tissue very problematic. qRT-PCR show that kininogen is more than 2 fold higher expressed in the adductor muscle of the ligated leg as compared to the unligated leg (Figure 19).

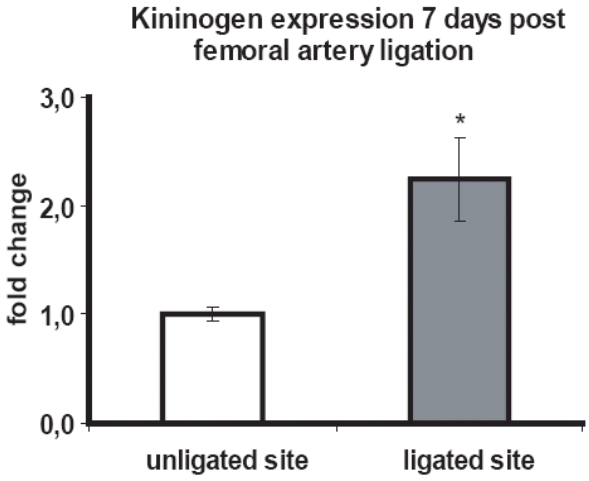

Figure 19. Kininogen expression in the mouse hind limb 24h post femoral artery ligation

Kininogen mRNA expression is upregulated in the adductor muscle tissue, which contains the collateral vessel region, in the ligated leg, as compared to the unligated leg.

3.3.2. Collateral growth following femoral artery ligation in bradykinin recetpor mutant mice

The capacity for collateral growth was measured by microsphere infusion 7 days after femoral artery occlusion (section 2.2.1.). Values obtained in the ligated left leg were normalized against the collateral conductance of the unligated right leg (100%) to evaluate arteriogenesis. The extent of peripheral arteriogenesis was measured in C57BL/6 wild type mice and compared with data obtained from different C57BL/6 bradykinin receptor KO mice strains. Data are shown in Figure 20. All bradykinin receptor KO mice strains exhibit a significantly reduced collateral capacity in comparison to the wild type mice.

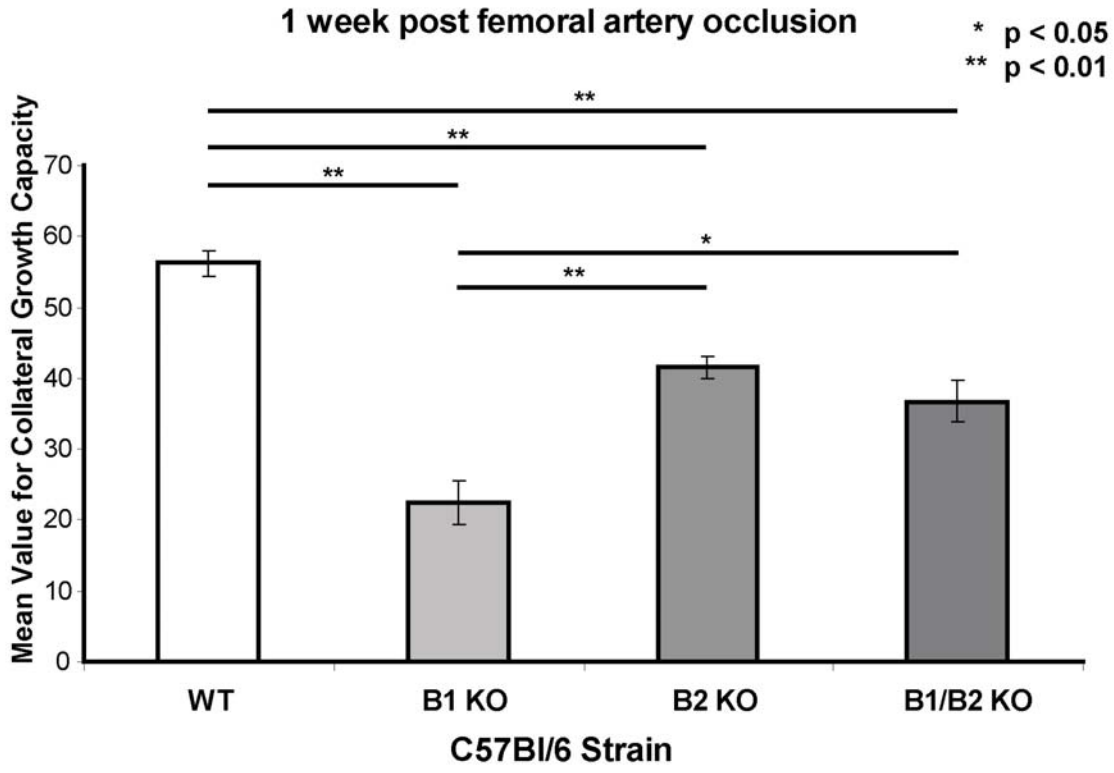

Figure 20. Degree of collateral capacity in bradykinin receptor KO mice
Collateralization is given for WT, B1 receptor KO, B2 receptor KO and B1/B2 receptor double KO 7 days post femoral artery occlusion. A degree for collateral capacity is measured under maximal vasodilation by pressure controlled infusion of fluorescent microsphreres of different size mice. Under pressure controlled conditions counting fluorescent microspheres serves as a value for assessing arteriogenesis. Microspheres within the ligated leg were counted by FACS analysis and normalized against reference samples and adjusted to the number of microspheres within the unligated leg (100 %). Here mean collateral capacity is given in relative units.

In particular, bradykinin receptor B1 KO mice (22.41 units) exhibit a more than 50 % reduced collateral capacity as compared to WT mice (56.17 units), and the difference between those two mice strains was about 33.76 units (Figure 20). Hence, the B1 KO

mice had the strongest phenotype and arteriogenesis seem to be severely reduced. The bradykinin receptor B2 mice (41.53 units) also exhibit a significantly reduced collateral capacity as compared to wild type mice. However, the differences in collateralization between bradykinin receptor B2 KO mice and wild type mice (14.64 units) is much lower, as compared to the difference of the collateralization between bradykinin receptor B1 KO mice and wild type mice.

Interestingly, for the bradykinin receptor B1/B2 double KO mice, collateral capacity is shown to be reduced by 19.43 units as compared to wild type mice (Figure 20). Hence, no cumulative reduced collateral capacity was found, and the phenotype of double receptor KO mice remains between the phenotype of the bradykinin B1 ad B2 receptor KO mice. As shown in figure 20, the difference of the double receptor KO mice is significantly higher as compared to the bradykinin B1 KO mice, but the differences to the bradykinin receptor B2 KO mice is not significant.

4. Discussion

This work intended to characterize the morphological and molecular patterns of early-phase cerebral arteriogenesis. Thus, results would enable us to identify novel target molecules that initiate arteriogenesis, which could contribute to the development of a noval therapeutic strategy to induce collateral growth. The 3-VO model was used, which is the first model to study cerebral arteriogenesis under controlled conditions (Busch, et al., 2003), and for which clinical relevance by therapeutically induced collateral growth has been shown (Schneeloch, et al., 2004).

First, data shown here identify the PCA as region of interest (growing collateral) and describe the morphological features of the PCA post 3-VO in the adult rat brain. Characteristic features of the arteriogenic process in the PCA were shown, such as vascular proliferation and monocyte invasion. Direct evidence was given for endothelial cell activation upon 3-VO by scanning electron microscopy (SEM) in growing collaterals of the brain.

Second, this work summarizes the molecular mechanisms, which underlie the initiation processes during early-phase cerebral arteriogenesis. For the first time it presents a comprehensive gene expression profile of early-phase collateral artery growth. Bioinformatic databases, functional annotation, and validation of expression data indentified TIMP-1 and kininogen as marker of collateral growth in the brain.

Third, this study gives direct evidence for the functional relevance of kininogen-bradykinin signaling during arteriogenesis, measured in the well-characterized model of femoral artery ligation in bradykinin receptor KO mice.

4.1. 3-VO model was successfully reproduced in this study

Collateral arteries in the brain offer a circumventing blood flow to maintain brain oxygenation and substrate supply when a primary vessel in the cerebral arterial tree is critically occluded. The circle of Willis is the most capable collateral system in the brain, providing a low-resistance link between the four main supplying arteries (two carotid arteries and two vertebral arteries) (Hossmann, 2003). To study collateral growth in the brain, it was necessary to reproduce the non-ischemic 3-VO model for cerebral arteriogenesis, which has previously been established by Busch et al (Busch, et

al., 2003). Cerebral arteriogenesis in the circle of Willis is induced by a combination of unilateral carotid artery and bilateral vertebral occlusion (3-VO). 3-VO lead to reduced cerebral blood flow (CBF) in the ipsilateral hemisphere of about 50 %, as compared to sham controls. Ongoing occlusion time did not increase CBF any higher (Figure 9). This result reflects the observation of Busch et al., who found that post 3-VO blood flow remained at the 50 % level, which is of crucial importance to induce adaptive arteriogenesis. Only a lasting reduction in CBF simulates the situation of hypoperfusion after occlusion of a major artery, which occurs previously to stroke. In fact, Busch et al. showed that despite reduced CBF of about 50 %, rats did not exhibit behavioral changes or disabilities. Likewise rats, which underwent 3-VO in our study, did not show any neurological deficits. Furthermore, Busch et al. already confirmed that rats do not have noticeable infarct areals post 3-VO, as obtained by histological evaluation of cryostat sections using cresyl violet staining, RhoB immunohistochemistry, and TUNEL.

4.1.1. ACA and PCA are recruited as collateral pathways post 3-VO

Continuous hypoperfusion leads to a redistribution of blood flow via the circle of Willis, basically by recruiting the ACA and PCA. Here, blood flow seemed to be redistributed mainly via the PCA in order to supply the hypoperfused area with blood. The PCA diameter increases within 7 days significantly by 37 %, as compared to sham controls (Figure 10). Hence, increased shear force generated by enhanced blood flow successfully induces cerebral arteriogenesis in the PCA. These results verify data obtained by Busch et al., however, in contrast to previous findings, here it was shown that both PCA regions, ipsilateral and contralateral of the carotid artery occlusion, significantly increased in diameter. Busch et al. showed that the vessel diameter of PCA contralateral increased only to a lesser degree. In fact, the strong bilateral PCA outgrowth observed in this study seems logical, since the PCA of both hemispheres has to be recruited as a collateral pathway in order to supply the hypoperfused area with blood. Furthermore, in our study blood flow seems to be redistributed as well via the anterior part of the circle of Willis. The ACA increases significantly in diameter within 7 days by around 32 µm. The anterior part as well as the posterior part interconnects the normal perfused area contralateral of the carotid occlusion with the ipsilateral hypoperfusion area (Figure 10). Blood flow is redistribution via the ACA as well as the PCA, which explains arteriogenesis observed in both brain regions. However, since recruitment of the PCA is the shorter way to supply the hypoperfusion area with blood

as compared to the ACA, blood flow is higher in the PCA according to the law of Hagen-Poiseuille (Figure 2), which indicates that blood flow is inversely proportional to the vessel length. A vessel having twice the length of another vessel (and each having the same radius) will have twice the resistance to flow. Therefore, blood flow via the PCA should be higher. Indeed, outgrowth of the PCA was larger and the PCA was chosen as area of interest for analysing morphological and molecular patterns of early-phase cerebral arteriogenesis.

4.1.2. Immunhistochemical characterization of early-phase cerebral arteriogenesis

The best time point for analysing gene expression of growing collateral vessels in the brain has so far not been known. Vessel growth becomes detectable only seven days after 3-VO in the PCA; however, immediately after vessel occlusion, increased flow through pre-existing anastomoses lead to an increase in vessel wall shear stress, which biomechanically activates endothelial cells (Nagel, et al., 1994; Sampath, et al., 1995). Subsequent growth processes occur in the collateral tissue, endothelial cells proliferate, and monocytes are recruited to the site of active remodelling (Arras, et al., 1998). To confirm early-phase cerebral arteriogenesis, the PCA was observed morphologically for these specific features of collateral growth 24 hours and 3 days post 3-VO. Histological analysis and staining for PCNA 24 hours after 3-VO (Figure 11) showed increased levels of proliferation within the endothelial cell layer of the PCA, which remain until 3 days post 3-VO. Only active cell proliferation can result in an increased PCA diameter as observed under maximal vasodilation seven days post 3-VO.

Using an antibody against CD68, monocyte invasion was detected 24h and 3d post 3-VO, (Figure 11) demonstrating monocyte invasion, which is another major feature of arteriogenesis visible in the PCA. This study, for the first time, shows a rapid recruitment of monocytes in brain arteriogenesis already at early time points: 24h post 3-VO, indicating that monocytes have a crucial role in the starting phase of collateral growth. Interestingly, previous studies of Buschmann showed that by application of the pro-inflammatory colony stimulating factor GM-CSF, cerebral arteriogenesis could be stimulated (Buschmann, et al., 2003). GM-CSF stimulated arteriogenesis, due to an increase in monocyte recruitment and by prolonging the life span of macrophages (Hossmann and Buschmann, 2005). Monocytes exert an essential paracrine function for adaptive arteriogenesis, as they enhance the remodelling process of collateral arterial

vessels (Hossmann and Buschmann, 2005). The functional relevance of monocytes for arteriogenesis was supported by a study from Heil et al., which provides a direct positive correlation between collateral artery growth and the number of circulating monocytes in the blood stream (Heil, et al., 2002). Finaly, the pre-dominant role of monocytes has been shown by two studies using the femoral artery ligation model analysing arteriogenesis in the periphery. A study, which aimed at blocking transendothelial migration of monocytes via ICAM inhibitory antibodies, demonstrated a severe reduction in collateral growth (Arras, et al., 1998). Proof of concept was given by the finding that osteopetrotic (op^-/op^-) mice, which have only minor populations of circulating monocytes, only partially recover from femoral arterial occlusion as compared to wild type mice (Bergmann, et al., 2006).

In summary, when combining previous findings with results obtained in this study, it appears that monocytes are recruited shortly (24h) after activation of endothelial cells by high blood flow for contributing to the initiation of cerebral arteriogenesis

4.1.3. Scanning electron microscopy shows distinct endothelial cell phenotypes in the PCA 3d post 3-VO

This study aimed at, for the first time, visualizing the morphology of the endothelial cell layer in growing collaterals of the brain after activation by high blood flow. The question arises: how structures of diminutive size, such as endothelial cells in a brain vessel could be ascertained in detail. In order to develop a novel technique, an interdisciplinary cooperation was started with the Institute of Protozoan Biology, Free University Berlin (Prof. Hausmann). Techniques, which were approved to image animalcule protozoen, would also be able to visualize the endothelial cell layer of a brain vessel. However, for preparing brain vessels for scanning electron microscopy, several techniques had to be tested for optimal tissue perfusion and staining to provide contrast to the image. Furthermore, different tissue glues were checked for feasibility to place the length-wise opened PCA on a glass slide. Critical point drying in liquid carbon dioxide, and subsequent spotting with ionized gold particles in an argon atmosphere succeeded in visualizing endothelial cell layer of the PCA. In conclusion, we managed to viszualize much smaller rat brain collaterals, as compared to collaterals of the dog heart, which were previously visualized by scanning electron microscopy by Schaper et al (Schaper, et al., 1972).

Scanning electron microscopy shows clearly, that 3d post 3-VO within the PCA leads to a cobblestone-like pattern of the endothelial surface, as compared to sham controls and align in the direction of flow. This finding is in line with findings of Schaper et al obtained by electron microscopy for early stages of collateral growth in the dog heart, who noticed that the formerly smooth endothelial layer becomes ‘‘rocky’’ (Schaper, et al., 1976). Schaper argued that endothelial cells loose cell-cell contacts to increase vessel permeability for subsequent monocyte invasion. It is reported that deformation of endothelial cells under high FSS is a typical feature of early arteriogenesis and is augmented by the intracellular edema by activation of ion channels and loss of volume control (Voswinckel, et al., 2003). *In vitro* studies also argued that endothelial cell activation can occur by opening of volume-regulated chloride channels or osmotic cell swelling via calcium influx (Schwarz, et al., 1992). It is speculated that this transformation of endothelial cells stress the cytoskeleton, which is required for gene activity typical for vascular remodelling (Ingber, 2002). Furthermore, the “rocky” structure, observed in this study by electron microscopy, may also displays a endothelial cell activation by flow changes characterized by nuclear protrusions (Figure 12). A protrusion of endothelial cells indicates that cells are turning to the mitotic cell cycle (McCracken, et al., 1979). This may explain why cells bulking into the lumen stain positive for proliferation marker PCNA 3d post 3-VO, as shown in Figure 11C; endothelial cells showed more pronounced structures on the cell surface and pronounced cell borders, compared to sham control animals, which might indicate a general change in gene expression. It may be also speculated that an increase production of junctional adhesion molecules after 3-VO is involved in a process of inflammatory cell recruitment, facilitating monocyte invasion into the proliferating vessel (Chavakis and Orlova, 2006). Furthermore, it is reported that endothelial cells upon elevated fluid shear stress produce cilia that protrude from the endothelial surface and float in the blood stream to sense blood velocity (Hierck, et al., 2008; Nauli, et al., 2008; Poelmann, et al., 2008). Maybe structures observed in this study on brain endothelial cells may represent the early formation of such organelles like cilia.

In summary, morphological analysis of early-phase cerebral arteriogenesis in the brain shows endothelial cell activation, cell proliferation, and monocyte invasion 24h and 3 days post 3-VO. Genomic profile analysis of these early events shall help to shed light on the molecular patterns involved in the starting processes of arteriogenesis.

4.2. Molecular patterns of early-phase cerebral arteriogenesis show related patterns between arteriogenesis and angiogenesis

The molecular mechanisms that initiate the enlargement of collateral vessels are not yet fully known. Little is known especially for the cerebral circulation. A prerequisite for an elaborate gene expression analysis for arteriogenesis research is a detailed knowledge of where to find, and how to selectively isolate collaterals. Morphological studies of early-phase cerebral arteriogenesis performed in this study, demonstrated many features of arteriogenesis in the PCA and identified a particular region of collateral growth in the brain. For this study, the ipsilateral posterior cerebral arteries and parts of the posterior communicating arteries were selectively extracted as indicated (Figure 13). This study, for the first time, provides a comprehensive gene expression analysis at induction of an arteriogenic stimulus (enhanced shear stress) by 3-VO, and provides important insights into the relationship of molecular expression patterns involved in the onset of collateral growth in the rat brain.

Most genes were found deregulated 24 hours post 3-VO in the growing PCA. Global gene expression profiles identified 91 genes as upregulated and 73 genes as downregulated, as compared to sham-operated groups. A massive amount of data was generated that implicates the problems, which would have accompanied non-hypothesis driven research. Therefore, molecular patterns were characterized to understand the relationship of deregulated genes.

First, Ingenuity network analysis was performed, which identified a superior expression network for cardiovascular system development as a molecular background for early-phase cerebral arteriogenesis. The superior network is characterized by six expression patterns (24h post 3-VO), which are functionally related (Figure 14) and include genes, which are reported to interact in cellular mobility, proliferation, growth as well as inflammatory response (Figure 21).

Second, Biological function annotation by the DAVID Bioinformatic database looking for Gene Ontology overrepresentations, again verifies processes of early phase arteriogenesis and identified biological themes for regulation of cell proliferation, cell-cell signaling, and cell differentiation (Figure 21).

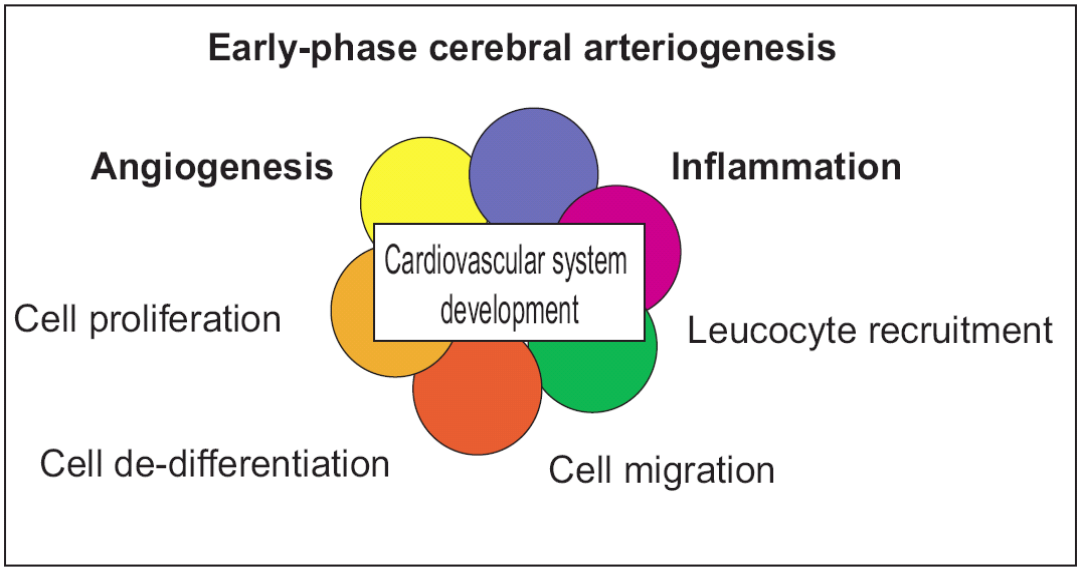

Figure 21. Scheme demonstrating the molecular expression patterns of early-phase cerebral arteriogenesis

Early-phase arteriogenesis is characterized by six functional goups, which all share characteristic features of cardiovascular system development. Functional annotation indicates that genes deregulated 24h post 3-VO are related to biological processes such as angiogenesis and inflammation. This implies relevant cellular processes such as cell proliferation, migration and de-differentiation, as well as leucocyte recruitment.

Patterns such as cellular growth, mobility, and proliferation correlate with the results obtained by immunhistochemistry and electron microscopy, showing endothelial cell proliferation and swelling. In fact, many genes annotated here to cell proliferation and migration, such as kininogen, semaphorin 4f, cell division cycle 2, and neurogenic differentiation 1, are published in the context of cancer, angiogenesis, and axon growth. Frequently, genes studied in the context of cancer are as well related to tumor angiogenesis, which is often a pre-requisite for tumor growth. Therefore, genes, such as kininogen, which releases bradykinin, feature a role for angiogenesis. Bradykinin is shown to promote angiogenesis by upregulation of bFGF through the B1 receptor or by stimulation of VEGF formation via the B2 receptor (Colman, 2006).

Genes involved in neuronal guidance and axon growth such as semaphorin 4f and neurogenic differentiation 1 could indicate a contamination by adjacent brain tissue, but much more likely those genes are related to angiogenesis and vascular remodelling such as arteriogenesis as well. Specialized endothelial cells, resembling axonal growth cones, form the tips of growing capillaries (Eichmann, et al., 2005). It is reported that blood vessels and nerves are structurally similar complex branched systems. The choreographed morphogenesis of both networks suggests that they are directed by

genetically programmed mechanisms. Several axon guidance molecules, including semaphorins, and ephrins, have also been implicated in vessel growth and angiogenesis (Carmeliet, 2005). In particular, semaphoring 3E and its receptor plexin D1 have recently been shown to direct endothelial tip cell navigation in sprouting angiogenesis (Eichmann, et al., 2005). Therefore genes annotated here to angiogenesis, involved in vessel and neuronal network patterning, are likely to be important candidates for cardiovascular system development in the context of endothelial cell proliferation and migration in early-phase arteriogenesis (Figure 21).

In summary, endothelial cell proliferation and survival is an essential mechanism during angiogenesis. Both processes also have a major impact for arteriogenesis, since vascular remodelling processes share major features (Carmeliet, 2000). Therefore, further study of genes listed in the appendix, which are related to proliferation, may disclose the differences of distinct mechanisms between angiogenesis and arteriogenesis.

4.2.1. Molecular patterns of early-phase cerebral arteriogenesis indicate that brain arteriogenesis takes place in an inflammatory environment

Early-phase cerebral arteriogenesis is characterized by a positive regulation of genes related to inflammatory response (Table 5). Expression of genes such *alpha 2-macroglobulin*, *chemokine ligand 14*, *complement component 1* and *3* and *lipopolysaccharid binding protein* are annotated to processes like leucocyte migration and inflammatory response (Appendix). These genes are likely to contributing to early processes, which leads to the initiation of collateral formation. To give some examples complement component C1q, the first subcomponent of complement system, initiate the expression of MCP-1 by human umbilical vein endothelial cells (van den Berg, et al., 1998). For MCP-1 signaling it was shown to attract monocytes and stimulate arteriogenesis in the rabbit periphery (Schirmer, et al., 2004). Second, complement component C3a is reported to be a chemoattractant for monocyte-derived dendritic cells as shown by Gutzmer et al, (Gutzmer, et al., 2004). Third, lipopolysaccharide binding protein is associated with TLR4 signaling, which results in NF-kB mediated inflammatory processes. Interestingly, for TLR4 it was shown to induce flow-induced outward remodelling (Timmers, et al., 2007). Furthermore, stimulation of collateral growth in the rabbit hind limb by LPS, a Toll-like receptor 4 ligand, attracts monocytes and markedly stimulated arteriogenesis (Arras, et al., 1998). In fact, this finding correlates well with the rapid invasion of monocytes in the perivascular space of the

PCA 24h post 3-VO, as observed by CD68 staining (Figure 11). Induction of expression of pro-inflammatory genes may be a key event for early arteriogenesis, which again is supported by previous findings that pro-inflammatory cytokines like GM-CSF stimulate arteriogenesis in the brain (Busch, et al., 2003). This result support the increasing evidence, that components of inflammatory responses constitute critically important players for initiating arteriogenesis (Arras, et al., 1998; Heil, et al., 2002). Analysing genes, deregulated in early-phase cerebral arteriogenesis related to monocyte migration and inflammatory processes, therefore may hold the opportunity for the development of a novel therapeutic stimulation of arteriogenesis.

Since expression profiles were obtained from material of isolated vessels in spatial distance from the surgical wound (extracranial ligation), detection of gene expression caused by post-operative inflammatory response seemed unlikely. Moreover, white blood cell count (Figure 16) and serum amyloid alpha levels (comparable to CRP in humans) were unaffected. Yet, it has to be considered that any injury and surgery can cause a post-operative inflammation, able to lead to an acute phase reaction.

Tzima showed that fluid shear stress triggers the conformational activation of integrins, which mediates both the alignment of endothelial cells in laminar shear and the activation of NF-kB in response to changes in shear (Tzima, et al., 2002; Tzima, et al., 2001; Tzima, et al., 2003). Lehoux et al. could show that high flow conditions for 24 hours subjected to human umbilical vein endothelial cells induce IkappaB alpha degradation and NFkappaB p65 phosphorylation, which activates MMP-2 and MMP-9 (Castier, et al., 2009).

Furthermore, by initiating complex signal transduction cascades, mechanical forces exerted on endothelial cells generate nitric oxide (NO), which leads to vasodilation and consequently results in stretching of smooth muscle cells in the vascular wall. Previous studies demonstrate that NF-kB is activated within smooth muscle cells by stretch as well (Lehoux, et al., 2006).

Candidate gene promoter analysis of the 53 most strongly deregulated genes identified 42 genes as having putative NF-kB binding sites and only 5 with SSRE. This result shows, that most genes may be regulated by NF-kB activation. In particular, the most strongly deregulated protease inhibitors are driven by NF-kB.

Hence, the overall transcriptional expression patterns indicate the following: shear stress exerted by high flow on endothelial cells may be the driving force, which activates

arteriogenesis, however, early-phase arteriogenesis 24-post 3-VO is governed by inflammatory processes and is associated with activation of NF-kB. NF-kB leads to the expression of pro-inflammatory genes and triggers inflammatory processes, such as monocyte transmigration into the perivascular space, which could be verified in this study. In summary, local inflammatory processes, which directly follow mechanotransduction by endothelial cells, still seem to be the most characteristic features reflecting the *in vivo* situation of collateral growth (Figure 21)

4.2.2. Similarities and differences of the molecular patterns of arteriogenesis in the periphery and the heart as compared to cerebral arteriogenesis

Molecular patterns obtained for early-phase cerebral arteriogenesis correlate with a pioneer work by Lee et al., who analysed global changes in gene expression of collateral growth in the mouse hind limb following femoral artery ligation. Lee et al. clustered genes deregulated in peripheral arteriogenesis according to similarity of temporal expression patterns. He identified an early induction phase in arteriogenesis with many genes deregulated 24h post femoral artery ligation, including genes associated with angiogenesis and inflammation. Genes that were upregulated at mid-phase, defined as those genes starting at day 3, included genes associated with cell cycle regulation, cytoskeletal-related genes, and additional inflammation-related genes. Therefore, molecular patterns identified herein are in accordance with the early to mid-phase induction seen by Lee at al. (Lee, et al., 2004).

Furthermore, Lee at al showed that genes encoding contractile proteins were either enhanced or repressed. Downregulation of genes encoding actin components was identified for early arteriogenesis in the periphery. For genes negatively regulated in our study, biological function annotation identified components of regulatory proteins of muscle development and contraction (Table 5B) in the growing PCA, such as smoothelin, myosin light chain, and calsequestrin 2. Downregulation of such components represent the initial step for smooth muscle cell (SMC) de-differentiation in processes of vascular growth. This finding is in line with results obtained by Schaper and co-workers (Schaper, 2004), who identified four phases of changes in endothelial and SMC phenotypes during collateral growth. Phase one and two are associated with early-phase arteriogenesis characterized by SMC de-differentiation. During phase one, SMCs turn from a contractile phenotype to an immature, synthetic phenotype, which allows proliferation and migration (Schaper, 2004).

The second phase is characterized by the controlled digestion of the extra-cellular matrix and internal elastic lamina synchronously with a burst of mitotic activity of SMCs and endothelial cells (Cai, et al., 2000; Scholz, et al., 2000). Studies performed on growing collaterals in the dog heart demonstrate that digestion of extracellular matrix is accompanied by the expression of MMPs and their inhibitor TIMP-1 (Scholz, et al., 2000). A close relationship between phenotype, migration, and proliferation of SMCs and the presence of MMPs (MMP-2 and MMP-9) and TIMP-1 has been reported *in vitro* and *in vivo* (Hultgardh-Nilsson, et al., 1997; Li, et al., 1996; Pauly, et al., 1994; Southgate, et al., 1992). The strong expression of TIMP-1 could be validated in this study and was localized by *in situ* hybridisation in the growing PCA 24h post 3-VO. Here enhanced expression of TIMP-1 may play an important role in keeping medial structures from disruption by limiting excess proteolysis (Dollery, et al., 1999; Forough, et al., 1996).

In this context it is important to mention the difficulty of comparing molecular data obtained here in this study with data obtained for arteriogenesis in the periphery or heart. Molecular patterns identified here are in accordance with previous studies, however, genes identified in detail frequently vary from the study of Lee and Schaper. Microarray data found by group of Schaper analysing gene expression of growing collateral vessels of the rat hind limb, showed a prominent role of the transient receptor potential cation channel, subfamily V, member 4 (Trpv4) (Troidl, et al., 2008). TRPV4 is activated by a variety of signals, like fluid shear stress and endothelial cell swelling (Vriens, et al., 2004). Here upregulation of TRPV4 in the growing PCA could not be verified. However, other genes upregulated in early-phase collateral growth of the mouse hind limb related to inflammation, such as MCP-1 could be detected as deregulated in this study (Appendix, Table 18).
Other genes expressed in other studies such as TIMP-1 correlate with the findings from previous studies. However, temporal expression pattern and localization differ in comparison to the expression data obtained in this study for the brain. For instance, in the dog heart TIMP-1 could only be detected in the late phase of remodelling specifically in the SMCs of collateral vessels. Schaper already suggests that the difference of gene expression probably resulted from different species, model, and sampling sites (Schaper, 2004). Thus, it is possible that each model of vascular injury or growth has its own features of remodelling through different regulating pathways.

Furthermore, the studies of Lee, and other studies performed by Schaper, dealt with the molecular mechanism of arteriogenesis in the periphery by applying the femoral artery occlusion model, which suffered from some major flaws (Boengler, et al., 2003), (Boengler, et al., 2003; Boengler, et al., 2003). The femoral artery occlusion model is a famous model for measuring blood flow and collateral conductance (performed in this study by approximation for bradykinin receptor KO mice). However, for analysing gene expression, this model exhibits two major disadvantages.

First, in the hind limb model, changes in gene expression occur in a region of active ischemia caused by femoral artery ligation. In contrast, 3-VO is performed extracranially and gene expression is analysed in a vessel far distal to the occlusion. Therefore, none of the deregulated genes found in this study are part of the response to local ischemia. A second limitation of the hind limb expression study was that the proximal portions of the developing collateral vessels are located within the skeletal muscle, and the whole adductor muscle was taken for RNA extraction.

Hence, isolation of collateral vessel tissue is cumbersome, since selective isolation (other than microdissection) of the latter often leads to contamination by adjacent muscle tissue (peripheral and heart models). In the 3-VO model, selective surgical removal and analysis of collateral vascular tissue for gene expression analysis without contamination by adjacent tissue could easily be performed (Figure 13). Therefore, the 3-VO model perfectly allows gene expression studies, rendering it the more suitable model for analysing gene expression, as compared to recent studies analysing arteriogenesis in the periphery.

4.2.3. Results of molecular analysis of cerebral arteriogenesis differ from results data obtained in *in vitro* studies

Shear stress is a fundamental determinant of vascular homeostasis regulating vascular remodelling, cardiac development, and atherogenesis, but its mechanisms of transduction are poorly understood. Mechanosensing pathways are required for the earliest-known events in arteriogenesis. Therefore, many *in vitro* studies aim at understanding the fundamental role of shear stress on different types of endothelial cells in order to identify target molecules, which initiate remodelling processes and are directly associated with mechonsensing. Studies analysing endothelial cell expression exposed to high levels of shear stress, show activation of the transcription factor Kruppel-like factor 2, which has been published to be an integrator of various mechanical and biologic stimuli (Dekker, et al., 2002; Fledderus, et al., 2007). Another

outstanding study by Tzima et al. identified a flow-induced mechanosensory complex comprised of PECAM-1 (which activates Src), VE-cadherin (which functions as an adaptor), and VEGFR2 (which activates PI(3)K) (Tzima, et al., 2005).

Neither KLF2 nor the compounds of the PECAM-1/VEGF-2 complex were found to be deregulated in our study. However, no great scale arteriogenenesis gene expression study (Schaper, Lee and others) has so far show deregulation of those components *in vivo*.

Genomic expression profiles of arteriogenesis generated from *in vivo* models yield complex expression patterns, indicating differential regulation of genes expressed in endothelial cells, smooth muscle cells and fibroblasts. In comparison, *in vitro* studies are artificial settings, and studies with endothelial cells under simulated flow conditions do not necessarily reflect the *in vivo* situation. Results change dramatically dependent on the stimulation with different compounds, by different types of cultured cells, and different flow simulations. This issue is highlighted by reports showing that FSS decreases the expression of MCP-1 in cultured endothelium, which is in contrast to the *in vivo* situation. Likewise, shear stress and nitric oxide seem to reduce monocyte attachment *in vitro*, but the exact opposite happens during collateral artery growth (Schaper, 2004; Tsao, et al., 1995; Zeiher, et al., 1995).

4.2.4. A novel role for protease inhibitors in early-phase arteriogenesis

Analysis of the identified expression networks for early-phase cerebral arteriogenesis by molecular function annotation showed lowest p-values for the protease inhibitors. The most upregulated genes include *kininogen* (66.4 fold change), *TIMP-1* (6.1 fold change), *A2M* (3.2-fold change), and *Lipocalin-2* (6.3 fold change). The latter is related functionally to TIMP-1, as revealed by network analysis (Figure 14).

Previously, TIMP-1 and MMPs were reported to be transcriptionally upregulated in a model of coronary arteriogenesis in the dog heart (Pipp, et al., 2004). In our study, *in situ* probes for TIMP-1 identified mRNA staining in the intima, media, and adventitia of the PCA during arteriogenesis 24h post 3-VO (Figure 18). Hence, TIMP-1 may be suggested as a marker for early-phase arteriogenesis in the brain. TIMP-1 controls matrix metalloprotease (MMP) activity (e.g. MMP-9 and MMP-2), thereby mediating extracellular matrix degradation by proteolysis, an important step during adaptive arteriogenesis (Cai, et al., 2000). MMPs are important mediators of remodelling and extracellular matrix degradation and regulate smooth muscle cell phenotype, proliferation, and migration, maintaining the integrity of the vascular wall (Kenagy, et

al., 1997; Kumar and Owens, 2003; Pauly, et al., 1994). However, in contrast to upregulation of TIMP-1, we could not detect regulation of MMPs on gene expression level in the early phase of arteriogenesis. This might be due to the fact that many MMPs are regulated on post-translational level. As shown by network analysis (Ingenuity), TIMP-1 is functionally related to Lipocalin 2 (LCN2). LCN2 can be expressed in blood vessels upon inflammation, forming a protein complex with MMP-9 and TIMP-1 (Bu, et al., 2006). Upon complex formation, degradation of MMP-9 by TIMP-1 is significantly inhibited, resulting in prolonged MMP-9 activity. The effect of Lipocalin 2 on MMP-9 activity was shown to be independent of changes in MMP-9 gene transcription (Fernandez, et al., 2005), which gives another possible explanation for unchanged MMP expression in early-phase cerebral arteriogenesis (Deindl, et al., 2001).

Besides MMP regulation, TIMPs were found to be involved in regulating cell proliferation, angiogenesis, and apoptosis (Mannello and Gazzanelli, 2001). Therefore, TIMP-1 might also mediate proliferation and protection from apoptosis in vascular cells during early phase arteriogenesis. Indeed, promoter analysis identified 9 NF-kB binding sites for TIMP-1, which is the highest number obtained for all genes analysed. A further role of protease inhibitors independent of their protease-inhibiting enzymatic function is therefore likely. Maybe TIMP-1 is regulated directly by inflammation to promote activation of vascular cell proliferation.

So far, A2M has not been reported in context with collateral artery growth. A2M is a protease inhibitor negatively regulating a broad range of proteases e.g. trypsin, thrombin, and MMPs, removing them by receptor mediated endocytosis (Baker, et al., 2002). Therefore, A2M might play a role during ECM degradation in arteriogenesis. In addition, a transporter function of A2M for growth factors and cytokines has been described, modulating cytokine signaling (Borth, 1992). *In silico* analysis demonstrated that A2M can bind the pro-arteriogenic platelet-derived growth factor-BB (PDGF-BB) (Crookston, et al., 1993; LaMarre, et al., 1991). A2M can serve as a reservoir for latent PDGF-BB, regulating cell migration and proliferation through binding to its receptor. Furthermore, A2M binds transforming growth factor-*β* (TGF-*β*), vascular endothelial growth factor (VEGF), and FGF-2; all of them exhibiting pro-arteriogenic properties (Lloyd, et al., 2005; van Royen, et al., 2002). Surprisingly, components of the PDGF-receptor beta and TGF-β 3 were downregulated, which needs to be further analysed.

Interestingly, kininogen was the most strongly upregulated gene. RNA i*n situ* hybridisation showed that kininogen is highly expressed at 24h post 3-VO selectively in

regions of the intima, media, and adventitia of the growing PCA (Figure 18). Kininogen as well is a cysteine protease inhibitor, preventing degradation of the extracellular vascular matrix by MMPs, thus orchestrating the homeostasis of the vessel wall. In kininogen deficient rats, severe aneurysm formation can be observed, pointing towards a role for kininogen in vascular remodelling (Kaschina, et al., 2004). However, as well as described for TIMP-1, kininogen is a multifunction protein involved in many cellular processes.

In the rat, T-kininogen seems most abundant and is expressed mainly during inflammation (Kageyama, et al., 1987), e.g. in the arterial wall after injection of LPS into rat aorta (Okamoto, et al., 1998), which correlates with the human LMW kininogen splice variant. LPS signals via the transmembrane receptor Toll like receptor 4, which activates NF-kB signaling. Promoter analysis performed in this study found 3 putative NF-kB binding sites, again highlighting its role in inflammatory processes.

In general, all kininogens are cleaved by distinct kallikreins to generate bradykinin and lys-bradykinin. Activation of the kinin system is of major importance for vascular biology and is involved in regulating vasodilation which counteracts the renin-angiotensin system (Madeddu, et al., 2007). Furthermore activation of the kinin-system by releasing bradykinin has been shown to promote angiogenesis, reduce cardiovascular ischemia and protect against stroke in rat and mouse models (Chao, et al., 2006).

Following the identification of morphological and molecular patterns of early-phase cerebral arteriogenesis, this study aimed at identifying novel key players for arteriogenesis. Literature screening identified kininogen and bradykinin signaling as a high potential target in the context of arteriogenesis research.

4.3. Direct evidence for Bradykinin signaling in arteriogenesis

Kininogen is the substrate for kallikrein, which liberates bradykinin. Bradykinin signaling is mediated by activation of the kinin B1 and kinin B2 receptors, leading to a multitude of cellular function, such as cell proliferation, increased endothelial cell permeability and leucocyte migration (Pesquero and Bader, 2006). Hence, bradykinin signaling exhibit some interesting features similar to those described for collateral growth, however, a role for bradykinin in arteriogenesis has not been demonstrated so far (section 1.8.).

4.3.1. Bradykinin receptor 1 and bradykinin receptor 2 KO mice show reduced arteriogenesis

Results obtained from collateral capacity measurements of bradykinin receptor B1, B2 and B1/B2 double KO show that all three mice strain exhibit reduced arteriogenesis. Hence, this study for the first time gives direct evidence of the functional relevance of bradykinin signaling in arteriogenesis.

Reduced arteriogenesis in bradykinin receptor B1 and B2 KO mice is a considerable finding, since both receptors are involved in diverse cellular processes, mediating their biological function via different signaling pathways. Actually, both kinin receptors have been shown to be involved in angiogenesis; promoting angiogenesis either directly or by stimulation of other paracrine substances (Granger, et al., 1994). Results demonstrated in this work that arteriogenesis shares genes and pathways similar to those reported for angiogenesis (Figure 21). However, for emphasizing a possible role of bradykinin signaling in arteriogenesis, the biology of both bradykinin receptors has to be discussed in more detail.

Bradykinin receptor 2 is constitutively expressed on various cell types, including endothelial cells, smooth muscle cells, nerve fibers, leukocytes, and mast cells (Calixto, et al., 2000). Stimulation of the bradykinin receptor 2, mediates activation of the Akt-eNOS-GSK3Beta pathway, which results in vasodilation and endothelial cell proliferation, which may directly induce reparative angiogenesis (Emanueli, et al., 2004; Yao, et al., 2008). In this work, quantification of the collateral growth displays a minor, but significant, reduction of arteriogenesis in B2 receptor KO mice. Hence, activation of the Akt-eNOS-GSK3Beta pathway possibly contributes to arteriogenesis, since endothelial cell proliferation is an integral part of the outgrowth of preexistent collateral arteries. Schaper et al. reported that signaling pathways for arteriogenesis involve the MAPKinases, the Rho-pathway, and eNOS dependent pathways, and addedd, that the latter two may be worth studying to design stimulators of arteriogenesis (Cai and Schaper, 2008).

It was found that B1 receptor KO mice exhibit a reduced arteriogenic capacity of about 50%, thus displaying a pronounced phenotype. The reduced capability of collateral growth in B1R KO mice corresponds with data obtained by Emanuelli et al., who identified significantly reduced angiogenesis and tissue perfusion in a model of hind limb occlusion in B1R KO mice (Emanueli, et al., 2002). Stimulation of the B1 receptor activates NF-kB along with the production of inflammatory cytokines that promote

angiogenesis by a paracrine effects (Krankel and Madeddu, 2009; Medeiros, et al., 2004). Therefore, the strong phenotype observed in B1 receptor KO mice is most likely explained by the leading role of this receptor for inflammatory processes. Lack of B1 receptor signaling in mice, reduces the production of pro-inflammatory cytokines, such as MCP-1 or GM-CSF, for which an important role in promoting arteriogenesis was shown. Furthermore, using B1 receptor-deficient mice, several studies showed reduced leucocyte transmigration, which depends on this receptor (McLean, et al., 2000; Schulze-Topphoff, et al., 2009). As described before (section 1.3. and 4.1.3.) transendothelial leucocyte migration, especially that of monocytes and the production of inflammatory cytokines are key events in promoting and enhancing collateral growth. This might explain the strong reduction in arteriogenesis in B1 receptor KO mice. Furthermore, it is reported that leucocytes carry both bradykinin receptors and kinins, strongly influencing leucocyte migration (Bertram, et al., 2007). Recent studies demonstrate that kinins exert potent chemoattractant effects on human CD133, CD34, and murine Lin cKit progenitor cells through a PI3K/Akt/eNOS-mediated mechanism (Krankel, et al., 2008). Furthermore, stimulation of leucoctyes with bradykinin stimulates the polymorphonuclear leukocyte accumulation and synthesis of cytokines (Ahluwalia and Perretti, 1996).

In contrast to constitutive bradykinin receptor 2 expression, there is little bradykinin receptor 1 expression in most tissues. Its expression on endothelial cells may be induced or enhanced only by cytokines in stressful states, such as inflammation (Marceau, et al., 1998). Here, recent studies show again the involvement of NF-kB in inducing mouse kinin B1 receptor expression (Merino, et al., 2005).

Analysis of molecular mechanisms of collateral growth shows local inflammatory processes during arteriogenesis. Induction of expression of B1 receptor and its critical role for inflammation seems likely.

Interestingly, activation of the constitutively expressed B2 receptor is also capable of inducing the expression of the B1 receptor (Phagoo, et al., 1999). Therefore, reduced arteriogenesis in B2 receptor KO mice may be a secondary effect and result from a delayed bradykinin receptor 1 expression. However, the cross-talk between Bradykinin receptors 1 and 2 seems to be much more complicated.

For instance, bradykinin, which acts basically via bradykinin receptor B2 as well, stimulates migration of human monocyte-derived dendritic cells in-vitro, indicating a

role of the bradykinin receptor 2 for inflammatory processes as well (Bertram, et al., 2007).

4.3.2. Bradykinin receptor 1 and 2 double KO mice demonstrate a recovered phenotype for arteriogenesis as compared to bradykinin receptor 1 KO mice

Given the role of leucocyte migration, cytokine production, endothelial cell permeability, and proliferation for the bradykinin receptor 1 and 2, one would assume that B1 receptor and B2 receptor double knock out mice exhibit the pronounced phenotype and strongest reduction in arteriogenesis. Surprisingly, arteriogenesis in B1 and B2 receptor double KO mice was hardly reduced to a larger degree, as compared to arteriogenesis in B1 receptor KO mice. In contrast arteriogenesis in the bradykinin receptor double KO mice was comparable to the B2 receptor KO mice. Thus, if the B2 receptor signaling pathway is blocked, in addition to impaired B1 receptor signaling, the arteriogenic ability recovers. Hence, it may be speculated that for signaling via the B2 receptor, two mechanisms are possible, one of which is pro-arteriogenic, the other anti-arterogenic.

Interestingly, similar results were obtained by Souza et al. in a different scientific context (Souza, et al., 2004). Here, the interaction between B1 receptor and B2 receptor was investigated in a model of intestinal reperfusion injury. First, Souza et al. analysed the role for each receptor alone in rat and later the role of bradykinin signaling for reperfusion injury using B1 receptor KO mice. In bradykinin receptor 1 KO mice, impaired B1 receptor signaling lead to reduced inflammatory injury after reperfusion, suggesting that pro-inflammatory signaling and leucocyte recruitment is decreased. Likewise, blocking the B2 receptor by the pharmacological compound Icatibant, results in reduced reperfusion injury and inflammation. This again indicated that activation of B1 receptor and B2 receptor may directly induce leukocyte migration by kinins, and both may also act on leukocytes and/or endothelial cells to release proinflammatory mediators. However, if the bradykinin 2 receptor pathway is blocked in addition to impaired B1 receptor signaling (similar to the situation in the B1/B2 receptor double KO mice), this again results in enhanced reperfusion injury.

This means that a concomitant B1 and B2 receptor absence and blockage demonstrate a recovery of the inflammatory process. Transferring this finding to results obtained for arteriogenesis in this study, indicates that recovery of the phenotype of collateral growth

in B1 and B2 double KO mice is dependent on the inflammatory process, which has been shown to be a driving force for arteriogenesis.

In summary, arteriogenesis in bradykinin receptor 1 and 2 double KO mice is stronger, as compared to B1 receptor KO mice, indicating a compensatory effect for inflammatory processes. Furthermore, data highlight a yet unknown interaction between B1 and B2 receptors and indicate a more complex crosstalk in inflammatory processes.

4.3.3. Transkriptome analysis demonstrate deregulation of kininogens, but not of bradykinin receptors in peripheral arteriogenesis

To assess whether kininogen is expressed at 24h post femoral artery ligation in growing collaterals as compared to the brain, adductor muscles were taken from hind limb ligated animals for RNA extraction. A 2-fold higher expression of kininogen in the adductor muscle of the ligated leg as compared to tissue from the unligated leg was detectable. Heterogeneous tissue blurs gene expression data obtained, but the difficult anatomy in the hind limb necessitates RNA isolation from adductor muscle. Therefore, expression values were expected to be relatively low. This may, on the other hand, explain that Lee et al did not find deregulation of kininogen in his genomic approach obtained in the mice periphery.

Molecular analysis of gene expression in growing collaterals did not show different expression of the B2 receptor. Expression of the B1 receptor was below threshold. For the human B2 receptor, it was shown to be regulated at protein level, and binding of BK results in rapid, receptor-mediated ligand internalization accompanied by loss of surface receptors (Marceau, et al., 1998), which may explain unchanged expression values. A marginal expression of the B1 receptor could be specific for endothelial cells or leucocytes which may be hidden in tissue which includes RNA from ECs, SMCs and fibroblasts as well.

4.4. Summary

In conclusion, this study presents a functional gene expression analysis as well as a comprehensive morphological analysis of collateral pathways in the brain in order to gain a more thorough insight into the initial molecular processes during cerebral collateral arteriogenesis.

3-VO leads to redistribution of blood flow via the circle of Willis, inducing arteriogenesis in the ACA and PCA. Induction of arteriogenesis is accompanied by biomechanical activation of endothelial cells in the PCA, resulting in vascular cell proliferation as early as 24 hours post 3-VO with increasing cell proliferation and nuclear protrusion in endothelial cells 3 days post 3-VO. Early-phase cerebral arteriogenesis is accompanied by macrophage adhesion and invasion into the perivascular space of growing collaterals. Following active cell proliferation, PCA diameters increase significantly within 7 days post 3-VO.

In line with these observations, early-phase cerebral arteriogenesis is characterized by the expression of genes involved in cell proliferation, cell migration, and cell de-differentiation. Genes are clustered in six functional related gene expression networks involved in cardiovascular system development. Here arteriogenesis is governed by genes, which are basically associated to the biological processes of inflammation and angiogenesis, notably by expression of protease inhibitors. It is demonstrated that protease inhibitors kininogen and TIMP-1 are potential biomarkers for cerebral arteriogenesis.

Enhanced expression of kininogen during early-phase arteriogenesis, exemplifies their multifunctional role, not only controlling extracellular matrix degradation, but also stimulating collateral growth by specific pathways. Functional relevance of kininogen-bradykinin signaling for collateral growth was ascertained using the femoral artery occlusion model in mouse. Arteriogenesis in the periphery was measured by microsphere perfusion. Seven days after femoral artery ligation in B1 receptor knockout (KO) mice, B2 receptor KO mice and B1 and B2 receptor double KO mice, arteriogenesis was significantly reduced among all bradykinin receptor mutant mice strains as compared to wild type animals. Here, bradykinin receptor 1 KO showed strongest reduction, and the phenotype of the B1 and B2 double KO mice indicate a more complex interaction of both kinin receptors for arteriogenesis, presumably in the context of inflammatory processes. However, further studies are needed to examine the role of kinins during early-phase cerebral collateral growth.

4.5. Study Limitation

Major foci of this study were to characterize the 3-VO model, analyse the molecular features of early-phase cerebral arteriogenesis, and identify kinin signaling as a novel candidate for arteriogenesis research. Here, especially for the kinin system, for which functional relevance was shown, this study clearly lacks of protein data.
However, much effort was invovled in performing immunhistochemistry to stain the PCA on brain sections for kininogen, B1 receptor 1 and bradykinin. Unfortunately no antibody against rat kininogen and bradykinin are available. Antibodies against other species were tested for cross-reactivity, but failed in obtaining any result. For staining kinin- components in the mouse hind limb, currently ongoing efforts are undertaken (Section 4.6).

4.6. Concluding remarks and Outlook

Myocardial infarction and stroke are the leading causes of mortality in developed countries. Ischemic disease affecting lower extremities represents a relevant clinical problem as well. Unfortunately, many patients cannot benefit from advances in revascularization techniques because of the extension of arterial occlusion. Consequently, there is a compelling need for alternative strategies. Collateral circulation can significantly influence occurrence and size of cerebral infarction (Liebeskind, 2004). Therefore, therapeutic stimulation of arteriogenesis might have important clinical implications for the development of prophylactic and acute treatments of vascular disease (Love, 2003).

In fact, recent studies demonstrate that activating the kinin pathway may protect against cardiac dysfunction and stroke (Chao and Chao, 2005; Yao, et al., 2008). Therapeutic value in the treatment of ischemic disease by promoting neovascularization was shown for the application of kinins (Smith, et al., 2008). This study demonstrates that kininogen is a marker of collateral growth in the brain. Furthermore it was shown, that kinin signaling is relevant for arteriogenesis. Thus, pharmacological manipulation of kinin receptors might open therapeutic options for stimulating arteriogenesis. However, further studies are needed to examine the mechanisms of kinin signaling to affect arteriogenesis.
Based on this study, a 3-year postdoctoral fellowship from the Center for Stroke Research (CSB), initiated by the Charité Berlin, and founded by the German Ministry of

Education and Research (BMBF) was awarded to the author to lay the basis for a strategy to induce cerebral arteriogenesis therapeutically as a preventive concept for stroke.

Future projects include, lose-of-function models and gain-of-function models, using the 3-VO rat brain model. Here, B1 receptor pathway and the B2 receptor pathway are pharmacologically inhibited by compound HOE-140 (Jerini AG) and compound H-1960 (Bachem). Currently, ongoing efforts are undertaken to analyse, in which cerebral arteriogenesis is stimulated by application of kinin-compounds. Furthermore, cerebrovascular reactivity as a functional parameter of cerebral arteriogenesis shall be assessed in rat selectively, overexpressing B1 receptor in endothelial cells. Application of bradykinin in animal models of ischemic stroke and subsequent measurement of the stroke volume, shall answer the question for a clinical relevance of kinin signaling for arteriogenesis.

Immunhistochemistry of the kinin system in the mouse hind limb post femoral artery occlusion, shall give evidence of protein expression in arteriogenesis. However, for staining of kinin- components in the mouse hind limb, the collateral region and region of interest need to be identified. Finally, collateral capacity following femoral artery occlusion in chimeric Bl6/mice after bone marrow transplantation from bradykinin receptor KO mice shall be measured. This shall answer the question whether collateral growth is regulated by the chemotactic homing of monocytes and other leucocyte sub-populations to areas of collateral remodelling by kinin signaling.

However, a major problem has to be considered: the so-called Janus phenomenon of arteriogenesis base on the observation that physiological (arteriogenesis) and pathophysiological (atherosclerosis) vascular remodelling share molecular features and are determined by common genetic programs. For instance, plaque formation in atherosclerosis, is accompanied by inflammation, cytokine production, and monocyte invasion. Therefore, therapeutic stimulation of arteriogenesis by pro-inflammatory cytokines may be inadequate for therapy, since it may have the potential to induce or increase atherosclerosis as well.

Atherogenic and arteriogenic predispositions seem both to be strongly influenced by the genetic background. Therefore, genomic studies have to be performed to understand the molecular differences contributing to arteriogenesis and atherogenesis. Kinin-mediated vascular growth and crosstalk between bradykinin receptor 1 and bradykinin receptor 2,

concerning the inflammatory response, might open new avenues for the selective stimulation of arteriogenesis.

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6. Appendix

Biological function annotation - upregulated genes

Table 8. GO-Categories Gene List 1 - Biological function annotation

response to wounding		inflammatory response	
AFFY_ID	**Gene Name**	**AFFY_ID**	**Gene Name**
1370281_at	fatty acid binding protein 5, epidermal	1367794_at	alpha-2-macroglobulin
1367794_at	alpha-2-macroglobulin	1388485_at	chemokine (c-x-c motif) ligand 14
1388485_at	chemokine (c-x-c motif) ligand 14	1368000_at	complement component 3
1368000_at	complement component 3	1387893_at	complement component 1, s subcomponent
1387893_at	complement component 1, s subcomponent	1387868_at	lipopolysaccharide binding protein
1387868_at	lipopolysaccharide binding protein	1370215_at	complement component 1, q subcomponent, beta polypeptide
1370215_at	complement component 1, q subcomponent, beta polypeptide	1367581_a_at	secreted phosphoprotein 1
1367581_a_at	secreted phosphoprotein 1	1387050_s_at	similar to alpha-1 major acute phase protein prepeptide
1398258_at	apolipoprotein d	1371079_at	fc receptor, igg, low affinity iib
1387050_s_at	similar to alpha-1 major acute phase protein prepeptide	1387088_at	galanin
1371079_at	fc receptor, igg, low affinity iib		
1387088_at	galanin		

Table 9. GO-Categories Gene List 2 - Biological function annotation

system development		cell differentiation	
AFFY_ID	**Gene Name**	**AFFY_ID**	**Gene Name**
1370281_at	fatty acid binding protein 5, epidermal	1368187_at	glycoprotein (transmembrane) nmb
1386879_at	lectin, galactose binding, soluble 3	1367581_a_at	secreted phosphoprotein 1
1368187_at	glycoprotein (transmembrane) nmb	1368926_at	semaphorin 4f
1370215_at	complement component 1, q subcomponent, beta polypeptide	1388802_at	brain expressed x-linked 1
1367581_a_at	secreted phosphoprotein 1	1367977_at	synuclein, alpha
1368926_at	semaphorin 4f	1389123_at	chemokine (c-c motif) ligand 6
1398258_at	apolipoprotein d	1387011_at	lipocalin 2
1388802_at	brain expressed x-linked 1	1370042_at	stathmin-like 2
1367977_at	synuclein, alpha	1387893_at	complement component 1, s subcomponent
1370042_at	stathmin-like 2	1367712_at	tissue inhibitor of metalloproteinase 1
1387893_at	complement component 1, s subcomponent	1367776_at	cell division cycle 2 homolog a (s. pombe)
1387131_at	serine (or cysteine) peptidase inhibitor, clade i, member 1	1369041_at	neuroligin 1
1367712_at	tissue inhibitor of metalloproteinase 1	1369152_at	protein phosphatase 3, regulatory subunit b, alpha isoform (calcineurin b, type i)
1371057_at	gamma-aminobutyric acid a receptor, alpha 5	1387088_at	galanin
1389250_at	lunatic fringe gene homolog (drosophila)		
1369041_at	neuroligin 1		
1370279_at	crystallin, alpha a		
1369152_at	protein phosphatase 3, regulatory subunit b, alpha isoform (calcineurin b, type i)		
1387088_at	galanin		

Table 10. GO-Categories Gene List 3 - Biological function annotation

negative regulation of cellular process		**regulation of cell proliferation**	
AFFY_ID	**Gene Name**	**AFFY_ID**	**Gene Name**
1372013_at	interferon induced transmembrane protein 1 (predicted)	1372013_at	interferon induced transmembrane protein 1 (predicted)
1387050_s_at	kininogen 1	1387050_s_at	kininogen 1
1368187_at	glycoprotein (transmembrane) nmb	1368187_at	glycoprotein (transmembrane) nmb
1367712_at	tissue inhibitor of metalloproteinase 1	1367712_at	tissue inhibitor of metalloproteinase 1
1367581_a_at	secreted phosphoprotein 1	1367581_a_at	secreted phosphoprotein 1
1387074_at	regulator of g-protein signaling 2	1388802_at	brain expressed x-linked 1
1367776_at	cell division cycle 2 homolog a (s. pombe)	1371079_at	fc receptor, igg, low affinity iib
1388802_at	brain expressed x-linked 1	1387088_at	galanin
1371079_at	fc receptor, igg, low affinity iib		
1367977_at	synuclein, alpha		
1387088_at	galanin		

Table 11. GO-Categories Gene List 4 - Biological function annotation

response to hormone stimulus		**regulation of multicellular organismal process**	
AFFY_ID	**Gene Name**	**AFFY_ID**	**Gene Name**
1367794_at	alpha-2-macroglobulin	1387050_s_at	kininogen 1
1369085_s_at	small nuclear ribonucleoprotein n	1368000_at	complement component 3
1370215_at	complement component 1, q subcomponent, beta polypeptide	1387893_at	complement component 1, s subcomponent
1367581_a_at	secreted phosphoprotein 1	1370215_at	complement component 1, q subcomponent, beta polypeptide
1370362_at	protein tyrosine phosphatase, receptor type, n	1367581_a_at	secreted phosphoprotein 1
1387088_at	galanin	1371079_at	fc receptor, igg, low affinity iib
		1387088_at	galanin

Table 12. GO-Categories Gene List 5 - Biological function annotation

mononuclear cell proliferation		leukocyte mediated immunity	
AFFY_ID	**Gene Name**	**AFFY_ID**	**Gene Name**
1387050_s_at	kininogen 1	1368000_at	complement component 3
1367581_a_at	secreted phosphoprotein 1	1387893_at	complement component 1, s subcomponent
1371079_at	fc receptor, igg, low affinity iib	1370215_at	complement component 1, q subcomponent, beta polypeptide
1387088_at	galanin	1371079_at	fc receptor, igg, low affinity iib

Table 13. GO-Categories Gene List 6 - Biological function annotation

adaptive immune response		organ development	
AFFY_ID	**Gene Name**	**AFFY_ID**	**Gene Name**
1368000_at	complement component 3	1370281_at	fatty acid binding protein 5, epidermal
1387893_at	complement component 1, s subcomponent	1386879_at	lectin, galactose binding, soluble 3
1370215_at	complement component 1, q subcomponent, beta polypeptide	1368187_at	glycoprotein (transmembrane) nmb
1367581_a_at	secreted phosphoprotein 1	1367712_at	tissue inhibitor of metalloproteinase 1
1371079_at	fc receptor, igg, low affinity iib	1370215_at	complement component 1, q subcomponent, beta polypeptide
		1367581_a_at	secreted phosphoprotein 1
		1371057_at	gamma-aminobutyric acid a receptor, alpha 5
		1398258_at	apolipoprotein d
		1389250_at	lunatic fringe gene homolog (drosophila)
		1370279_at	crystallin, alpha a
		1369152_at	protein phosphatase 3, regulatory subunit b, alpha isoform (calcineurin b, type i)

Biological function annotation - downregulated genes

Table 14. GO-Categories Gene List 7 - Biological function annotation

muscle development		**muscle system development**	
AFFY_ID	**Gene Name**	**AFFY_ID**	**Gene Name**
1370937_a_at	integrin alpha 7	1368988_at, 1387401_at	calsequestrin 2
1387969_at	chemokine (c-x-c motif) ligand 10	1370033_at	fast myosin alkali light chain
1390827_at	mad homolog 3 (drosophila)	1373915_at	dystrophia myotonica kinase, b15 (predicted)
1372684_at	smoothelin	1372684_at	smoothelin
1388876_at, 1370157_at	phospholamban	1388298_at	myosin, light polypeptide 9, regulatory (predicted)
1388298_at	myosin, light polypeptide 9, regulatory (predicted)		

Table 15. GO-Categories Gene List 8 - Biological function annotation

circulatory system process		**embryonic development**	
AFFY_ID	**Gene Name**	**AFFY_ID**	**Gene Name**
1370033_at	fast myosin alkali light chain	1390827_at	mad homolog 3 (drosophila)
1373915_at	dystrophia myotonica kinase, b15 (predicted)	1372299_at	cyclin-dependent kinase inhibitor 1c (p57)
1369664_at	arginine vasopressin receptor 1a	1389341_at	plexin d1 (predicted)
1388876_at, 1370157_at	phospholamban	1367674_at	phosphatidylinositol transfer protein, beta
1368154_at	guanylate cyclase 1, soluble, alpha 3	1367859_at	transforming growth factor, beta 3
		1373032_at	musculoskeletal, embryonic nuclear protein 1

Table 16. GO-Categories Gene List 9 - Biological function annotation

regulation of cell proliferation		organ development	
AFFY_ID	**Gene Name**	**AFFY_ID**	**Gene Name**
1386860_at	milk fat globule-egf factor 8 protein	1368988_at, 1387401_at	calsequestrin 2
1387969_at	chemokine (c-x-c motif) ligand 10	1370937_a_at	integrin alpha 7
1390827_at	mad homolog 3 (drosophila)	1370291_at	pdz and lim domain 3
1372299_at	cyclin-dependent kinase inhibitor 1c (p57)	1387969_at	chemokine (c-x-c motif) ligand 10
1376066_at	ras homolog gene family, member e	1390827_at	mad homolog 3 (drosophila)
1367859_at	transforming growth factor, beta 3	1372684_at	smoothelin
1372606_at	cell division cycle associated 7	1389341_at	plexin d1 (predicted)
		1367859_at	transforming growth factor, beta 3
		1388876_at, 1370157_at	phospholamban
		1373032_at	musculoskeletal, embryonic nuclear protein 1
		1388298_at	myosin, light polypeptide 9, regulatory (predicted)

Table 17. GO-Categories Gene List 10 - Biological function annotation

system development		**positive regulation of cellular process**	
AFFY_ID	**Gene Name**	**AFFY_ID**	**Gene Name**
1368988_at, 1387401_at	calsequestrin 2	1386860_at	milk fat globule-egf factor 8 protein
1387969_at	chemokine (c-x-c motif) ligand 10	1387969_at	chemokine (c-x-c motif) ligand 10
1372299_at	cyclin-dependent kinase inhibitor 1c (p57)	1390827_at	mad homolog 3 (drosophila)
1372684_at	smoothelin	1369942_at	actinin alpha 4
1389341_at	plexin d1 (predicted)	1376066_at	ras homolog gene family, member e
1370057_at	cysteine and glycine-rich protein 1	1367859_at	transforming growth factor, beta 3
1367859_at	transforming growth factor, beta 3	1369664_at	arginine vasopressin receptor 1a
1388876_at, 1370157_at	phospholamban	1371221_at	oxidative stress induced
1373032_at	musculoskeletal, embryonic nuclear protein 1	1387027_a_at	lectin, galactose binding, soluble 9
1370937_a_at	integrin alpha 7		
1370291_at	pdz and lim domain 3		
1390827_at	mad homolog 3 (drosophila)		
1388298_at	myosin, light polypeptide 9, regulatory (predicted)		

Table 18. Example of target gene validation by qRT-PCR

24h post 3-VO vs. 24h post sham surgery	**Fold change**	**p-value**
Complement Component C1Q beta	2.54	< 0.05
Lipopolysaccharide binding protein	2.36	< 0.05
glycoprotein (transmembrane) nmb	3.7	< 0.05
Phosphatidylserine-specific phospholipase A1	4.17	< 0.05
MCP-1 - Monocyte chemoattractive molecule 1	2.73	< 0.05

Excert of target genes validated**.** Expression is given as fold change as compared to sham controls.

Table 19. Vessel diameter mean values

Vessel Diameter in µm	MW 7d Sham	SEM	MW 7d 3-VO	SEM
PCA ipsi	159 µm	9.0	199 µm	9.9
PCA contra	143 µm	8.8	189 µm	11
MCA ipsi	199 µm	6.4	199 µm	7.8
MCA contra	186 µm	5.4	191 µm	7.8
ACA ipsi	165 µm	6.7	198 µm	7.8
ACA contra	158 µm	5.0	188 µm	7.8

Table 20. White blood cell mean values

White Blood Cell Count	MW	SEM
Untreated	9.53	0.36
24h Sham	10.07	1.58
24h 3VO	8.64	0.78

Table 21. Collateral conductance mean values

Collateral Conductance	Mean	SEM
WT	56.17	1.72
B1 KO	22.41	3.04
B2 KO	41.53	1.48
B1/B2 KO	36.74	2.87

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2. Abstracts

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Direct Evidence for Bradykinin signaling during early phases of arteriogenesis: Proof-of-concept study in B1 / B2 and double knock-out mice.

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Poster

Hillmeister P, Witt H, Dülsner A, Patricia Ruiz, Buschmann IR, Lehmann K
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2. Poster

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Identification of protease inhibitors Kininogen and TIMP-1 as novel markers of early-phase cerebral arteriogenesis

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Eidesstattliche Erklärung

Hiermit erkläre ich, die Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfen und Hilfsmittel angefertigt zu haben.

Ich habe mich anderwärts nicht um einen Doktorgrad beworben und besitze einen entsprechenden Doktorgrad nicht.

Ich erkläre die Kenntnisnahme der dem Verfahren zugrunde liegenden Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin vom 01. September 2005.

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(Philipp Hillmeister)